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# PURIFICATION AND CHARACTERIZATION OF DNA-DEPENDENT RNA POLYMERASE FROM VEGETATIVE CELLS AND BACTEROIDS OF *RHIZOBIUM JAPONICUM*

By

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF DNA-DEPENDENT RNA POLYMERASE FROM VEGETATIVE CELLS AND BACTEROIDS OF RHIZOBIUM JAPONICUM

By

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RNA polymerase was purified from both the vegetative and symbiotic forms of Rhizobium japonicum strain 3I1b 110 using DNA-cellulose and DEAE-cellulose chromatography. The physical and transcriptional properties of the purified enzyme from both sources were analyzed. The subunit composition of the enzyme from both sources appeared similar when analyzed by SDS-polyacrylamide gel electrophoresis and corresponded to the $\beta\beta'\alpha_2\sigma$ subunit structure of many procaryotic RNA polymerases. Removal of an 82 K protein from the purified enzyme from either source, using procedures developed for the isolation of the sigma factor from Escherichia coli RNA polymerase, resulted in a decreased ability to utilize T4 DNA as a template in vitro. These results indicate that the 82 K protein may act as a sigma-like factor for RNA polymerase of R. japonicum. The transcriptional specificity of the enzyme from both sources was similar using a variety of exogenous templates. There were also no apparent differences in the kinetic properties of the enzyme from either source or the specificity of promoter

utilization when T7 DNA was used as a template. RNA polymerase from both sources appeared to utilize *nif*-specific promoters from *Rhizobium japonicum* and *Klebsiella pneumoniae in vitro*. This indicates that no positive regulatory factor is necessary for the transcription of these genes *in vitro*.

To my parents

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ALA | amino-levulinic acid |
| BSA | bovine serum albumin |
| DEAE | diethylaminoethyl |
| DEPC | diethylpyrocarbonate |
| EDTA | (ethylenedinitrilo)-tetraacetic acid |
| EtBr | ethidium bromide |
| *fix* | nitrogen fixation |
| μCi | micro Curie |
| μE | micro Einstein |
| *nif* | structural genes for nitrogenase |
| *nod* | nodulation |
| POP | 2,5-diphenyloxazole |
| POPOP | 1,4-bis 2-(4-methyl-5-phenyloxazolyl) -benzene |
| PVP | polyvinylpyrrolidone |
| SDS | sodium dodecyl sulfate |
| *sym* | symbiotic |
| TCA | trichloroacetic acid |
| Tris | Tris(hydroxymethyl)aminomethane |

# INTRODUCTION

Bacteria of the genus Rhizobium are capable of establishing a species specific symbiotic relationship with legumes. Within the plant roots, vegetative bacteria undergo a process of cellular differentiation to form symbiotic cells referred to as bacteroids which carry out the process of biological nitrogen fixation. During this process of differentiation, a number of new gene products are induced or derepressed. These are required for the process of biological nitrogen fixation in the mature bacteroid and include the nif gene products (1), the cytochromes $c_{550}$, $c_{552}$, P-420, P-428 and P-450 (2), and the heme biosynthetic enzymes (3). In addition, the bacteria also undergo changes in cellular metabolism (4) and fail to synthesize certain cytochromes which are present in the vegetative state (5). The bacteria code for all of the gene products necessary to reduce nitrogen to ammonia (6-8), however, the expression of the nif and symbiotic genes normally occurs only in the symbiotic state.

The transcriptional control mechanism(s) which is responsible for the selective expression of these genes in the symbiotic state is, as yet, unknown. As a first step in analyzing the transcriptional control of the nif and symbiotic genes in vitro, I have purified RNA polymerase from both vegetative and symbiotic forms of R. japonicum. This enzyme was characterized to determine if any gross structural differences existed between RNA polymerase isolated from vegetative cells or bacteroids. New or modified forms of RNA polymerase have been shown to be responsible for

the transcription of specific genes required for the development of a number of bacteriophage (9) and in the process of sporulation in Bacillus subtilis (10). Since the development of the Rhizobium-legume symbiosis requires the transcription of a specific subset of genes which are essential for cellular differentiation and nitrogen fixation, this developmental process may be analogous to other systems which have been shown to involve novel forms of RNA polymerase.

The transcriptional properties of RNA polymerase from both cell types were also examined. The elongation rate, efficiency of termination, and promoter utilization of the enzyme from both sources were measured using T7 DNA as a template (11). The ability of RNA polymerase from both cell types to recognize nif-specific promoters was investigated. This information should be useful as a basis for studying the transcriptional regulation of genes necessary for the establishment of the Rhizobium-legume symbiosis in vitro.

The dissertation is divided into two chapters, corresponding to two general areas of research. Chapter I describes the purification of RNA polymerase from both vegetative cells and bacteroids of R. japonicum and a physical characterization of the enzyme. Chapter II describes a transcriptional characterization of the enzyme using both exogenous templates and nif-specific genes to characterize the transcriptional specificity of RNA polymerase from both forms of R. japonicum.

# LITERATURE REVIEW

## Rhizobium genetics and bacteroid development

Biological nitrogen fixation is the process by which atmospheric $N_2$ is enzymatically converted to ammonia. Procaryotes capable of carrying out this process can be divided into two groups: 1) free-living bacteria which utilize the newly fixed nitrogen for their own growth and 2) bacteria which fix nitrogen when symbiotically associated with plants. Bacteria of the genus Rhizobium are members of the second group and are capable of forming a species-specific symbiotic relationship with legumes.

The formation of legume root nodules results from a series of interactions between the appropriate Rhizobium species and its host plant (for review, see 12). The bacteria bind to the proximal end of the root hair, possibly through a lectin-mediated interaction, and infect the plant root through an infection thread of plant origin. The bacteria divide within the infection thread as it branches and penetrates through cells within the root cortex. The growth of the infection thread is correlated with plant cell division within the root cortex. As the nodule develops, the bacteria are released into the cytoplasm of target cells and are surrounded by the peribacteroid membrane. After being released from the infection thread the bacteria differentiate into bacteroids which are responsible for the fixation of nitrogen. The ammonia which is produced by the

bacteroids diffuses into the plant cytoplasm where it is assimilated into amino acids. In return, the plant supplies the bacteria with all of their nutrient requirements.

During the process of bacteroid development, the induction and/or depression of a number of new gene products is required for the process of nitrogen fixation in the mature bacteroid. These include the nitrogenase (nif) gene products (1), several new cytochromes (2) and the heme biosynthetic enzymes (3). In addition, the bacteria also undergo changes in cellular metabolism (4) and fail to synthesize certain cytochromes which are present in the vegetative state (5).

Nitrogenase, the enzyme complex responsible for the reduction of $N_2$ to ammonia, is composed of two soluble proteins. Component I is an FeMo protein which is composed of two copies each of two subunits ($\alpha_2\beta_2$) and which contains the catalytic center for the reduction of $N_2$ (13,14). An FeMo cofactor (FeMoCo) is also associated with the nitrogenase enzyme. This cofactor is a small molecule with a molecular weight of less than 5000 (15) and it is apparently an active site for the reduction of $N_2$ (16). However, isolated FeMoCo will not support $N_2$ fixation itself. Component II, nitrogenase reductase, is an non-heme Fe protein composed of two copies of a single subunit and functions by supplying electrons to Component I (17,18).

Both components of nitrogenase are highly susceptible to inactivation by $O_2$ (19). Legume root nodules synthesize large amounts of leghaemoglobin which binds free $O_2$, preventing it from inactivating the nitrogen-fixing apparatus. Leghaemoglobin is produced through an interaction between the bacteria and the host plant. The bacteria produce the heme moiety as a result of a derepression of the heme biosynthetic enzymes, ALA-

synthetase and ALA-dehydrase (3), while the plant synthesizes the globin apoprotein (21).

The bacteroid synthesizes the cytochromes $c_{550}$, $c_{552}$, P-420, P-428 and P-450 which are not made in the vegetative bacteria (2). These cytochromes appear to operate more efficiently at the low oxygen tensions which exist in the nodule and function in the synthesis of ATP which is needed to support the energy intensive process of nitrogen fixation. At the same time, certain cytochromes present in vegetative cells are not synthesized in bacteroids (5).

The development of the symbiosis requires the synthesis of gene products involved not only in the fixation of nitrogen but also in the recognition of the specific host plant and the differentiation of a bacterium into a mature bacteroid. Studies on the symbiotic properties of auxotrophic mutants and mutants resistant to phage and antibiotics have been used to identify possible developmentally regulated genes which function in the Rhizobium-legume symbiosis (for review, see 21).

The differentiation of vegetative bacteria into bacteroids appears to be a pre-requisite for symbiotic nitrogen fixation in many Rhizobium species. This involves cell wall alterations leading to changes in shape and cell wall structure (22). Mutants resistant to phage (23,24) and to antibiotics known to inhibit cell wall synthesis (25) have been examined for their symbiotic properties. In both cases, a large number of the mutants tested were $Fix^-$ in phenotype. The viomycin-resistant mutants in these studies were analyzed biochemically and were found to have an altered cell wall composition. It is possible that the altered cell wall composition of these mutants interferes with the physical changes in cell wall structure necessary for bacteroid development. However, a more ex-

tensive examination of cell wall properties in Rhizobium is necessary before any strong conclusions can be reached.

The isolation of mutants resistant to aminoglycoside antibiotics, such as neomycin, has been used to identify ATPase mutants in R. trifolii. These mutants were unable to use succinate as a sole carbon source, had reduced ATPase activity and were unable to synthesize ATP (26). Revertants were able to utilize succinate for growth, regained ATPase activity and were sensitive to neomycin, indicating that a mutation in a single gene was responsible for the mutant phenotype. When the symbiotic properties of the mutants were examined they were all found to be $Nod^{+}Fix^{+}$. Bacteroid preparations from these nodules had 70% of the wild type ATPase level, although they were still unable to metabolize succinate and were neomycin sensitive. These data suggest that a new ATPase may be induced during bacteroid differentiation.

Several auxotrophic and/or antimetabolite-resistant mutants of Rhizobium have been shown to have altered symbiotic properties. Riboflavin-requiring mutants of R. trifolii have been found to be $Fix^{-}$, and this mutation can be circumvented by the addition of riboflavin to clover plants infected with this mutant (27,28). Adenine auxotrophs appear to be symbiotically defective in several strains of Rhizobium; however auxotrophs of many other Rhizobium species exhibit major differences in regards to their ability to form effective nodules (29,30). This difference may be due to the nature of the metabolites that various legumes provide for the infecting bacteria (31).

While it appears that Rhizobium is incapable of assimilating newly fixed $N_2$ into amino acids (32), there is some evidence to suggest that glutamine synthetase plays a role in the regulation of nitrogen fixation.

Mutants in glutamine synthetase in R. sp. strain 32H1 (33) and R. meliloti (34) have been found to be ineffective in nitrogen fixation. However, the mechanism by which glutamine synthetase exerts its regulatory role is unknown.

Mutants in carbon metabolism have also been analyzed in an attempt to determine what carbon source(s) is provided by the host plant to drive the process of nitrogen fixation. R. trifolii mutants deficient in a number of enzymes necessary for hexose metabolism have been analyzed (35) including glucokinase, fructose uptake, pyruvate carboxylase and enzymes of the Entner-Douderoff pathway and all mutants tested were able to nodulate clover and fix nitrogen. Dicarboxylic acid transport mutants of R. trifolii and R. leguminosarum have been isolated and the symbiotic properties of the R. trifolii mutants have been tested (36,37). The dicarboxylic acid transport system was found to be inducible in both Rhizobium species and mutants of R. trifolii lacking this system were ineffective. This would indicate that dicarboxylic acids may be supplied by the host plant and used by the bacteroid to drive the process of nitrogen fixation. However, it does not rule out the possibility that other carbon sources may be supplied to the differentiating bacteria at earlier stages of development.

Although the bacteria have been shown to code for all of the gene products necessary to reduce $N_2$ to ammonia (6-8), the expression of the nif and other symbiotic genes normally occurs only in the symbiotic state. Recently, large plasmids in several Rhizobium species have been shown to code for nif and other symbiotic functions. The structural genes for nitrogenase have been located on large plasmids in strains of R. trifolii R. leguminosarum (39), R. meliloti (40,41), R. phaseoli (42), and R. japonicum (43). These large plasmids have also been shown to code for at

least some of the genes necessary for nodulation in a number of Rhizobium species (40,41,44,45,46). Krol et al. (47) have demonstrated that the large plasmids present in R. leguminosarum containing the nif genes are heavily transcribed in bacteroids and not in vegetative bacteria. However, the transcriptional control mechanism(s) which is responsible for the selective expression of these genes in the symbiotic state is, as yet, unknown.

## nif genetics and regulation in Klebsiella pneumoniae

The nif region in Klebsiella pneumoniae has been carefully characterized using both physical and genetic techniques (for review, see 48,49). The size of the nif region is 24 kb and it contains the coding sequences for at least 18 polypeptides. These genes are organized into 7 distinct operons and transcription of all of the genes is in one direction, toward the his gene (Figure 1).

The identity of many of the nif gene products and their functions are known. This information is summarized in Table I. The nif D and K genes code for the $\alpha$ and $\beta$ chains of component I and the nif H gene codes for component II of nitrogenase (50). Proteins coded for by nif M and S appear to be involved in the processing of component II (50). The function of the nif V and U gene products is not known, but it has been suggested that they might be involved in the modification of component II due to their cotranscription with nif M and S (51). Several gene products have been implicated in the synthesis and processing of the iron-molybdenum cofactor (FeMoCo) including nif B, Q, N, E and C (50,52,53). The products of nif F and J are involved in electron transport to nitrogenase (50,53). The nif F product is a flavoprotein which is only synthesized under $N_2$-fixing condi-

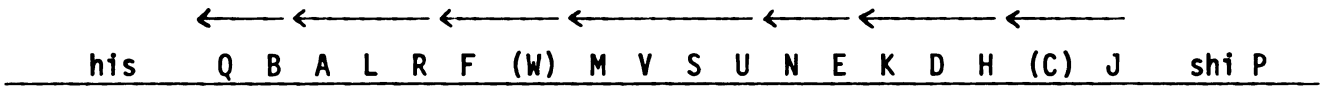



Figure 1 Order and transcriptional organization of Klebsiella pneumoniae nif genes
The 7 operons and the direction of transcription is indicated by the arrows above the genes listed (49).

Table I *Klebsiella pneumoniae nif* gene products[1]

| Gene | Molecular Weight | Function |
|---|---|---|
| Q | | (FeMoCo) |
| B | | FeMoCo |
| A | 66 | positive regulator |
| L | 52 | negative regulator |
| R | | ammonia regulation |
| F | 22,26 | electron transport |
| (W) | | |
| M | 27 | processing component II |
| V | 38 | (processing component II) |
| S | 42 | processing component II |
| U | 28 | (processing component II) |
| N | 50 | FeMoCo |
| E | 46 | FeMoCo |
| K | 60 | β subunit of component I |
| D | 56 | α subunit of component I |
| H | 35 | component II |
| (C) | | (FeMoCo) |
| J | 120 | electron transport |

[1] Information taken from Roberts, G.P. and W.J. Brill (1981), (49).

tions (54). The nif J protein is thought to be a reduced nicotinamide adenine dinucleotide phosphate flavoprotein reductase (55,56). Synthesis of the nif F protein appears to be under control of nif J (50).

DNA sequence homology existing between nif genes in Klebsiella and other nitrogen-fixing organisms has been examined (57). Cloned segments of the nif region of K. pneumoniae have been hybridized to DNA from a variety of nitrogen-fixing bacteria. In all cases, only a portion of the nif KDH operon was found to share sequence homology with nif DNA from other organisms. No sequence homology was found with DNA isolated from non-nitrogen-fixing microorganisms.

The regulation of expression of the nif genes in K. pneumoniae appears to be controlled by the nif RLA cistron (49). The nif A product is a positive regulatory factor which is necessary for the transcription of all nif operons other than its own (58). The nif L product acts as a negative regulatory factor at the post-transcriptional level and may be involved in the regulation of nif genes by $O_2$ (58). Proteins involved in the general nitrogen metabolism of the cell also appear to exert regulation on the expression of nif genes in Klebsiella. The mechanism of regulation is still unclear but it is thought to involve the products of at least 3 genes, gln F, gln L and gln G, and occur at the level of transcription (59, 60). Regulation seems to occur by interaction of these proteins with the nif R region, since mutations in this region renders the expression of the nif genes independent of the general nitrogen regulatory system (58). The gln A gene product has been shown to be able to substitute for the gln G gene product, and may also be involved in the control of nif expression in Klebsiella pneumoniae (61).

RNA polymerase in procaryotic systems

The transcription of genetic information in bacterial systems is mediated by DNA-dependent RNA polymerase. This enzyme has been purified from a number of bacterial sources (62). The enzyme from E. coli has been analyzed extensively and serves as a model for the characterization of other bacterial RNA polymerases.

The E. coli RNA polymerase holoenzyme is a $Zn^{+2}$-metalloenzyme (63) which contains 4 major subunits, $\beta'$, $\beta$, $\sigma$ and $\alpha$ in a ratio of 1:1:1:2 (64,65). The enzyme has a protomer molecular weight of 480,000-500,000 daltons and reversibly forms a dimer at low ionic strength (66). The protomer holoenzyme appears to function as the basic unit of selective transcription in vivo and is sufficient to carry out transcription in vitro (for review, see 9).

Both genetic and biochemical evidence have established that the $\beta'$, $\beta$, $\sigma$ and $\alpha$ polypeptides are functional subunits of RNA polymerase. The $\beta'$ subunit is the largest (160 Kd) and the most basic of the 4 polypeptides (64). It can bind polyanions, such as heparin (67), and is the only subunit which can bind DNA by itself (67,68). Bacterial mutants have been generated which have an altered $\beta'$ subunit (69-71). The RNA polymerases containing these altered subunits have impaired DNA-binding properties and are defective in recognizing transcription termination sequences (72).

The $\beta$ subunit is the second largest (150 Kd) of the 4 major RNA polymerase polypeptides and is thought to contain the catalytic center of the enzyme (64,73). This subunit has been shown to bind rifampicin (73) and streptolydigin (74), which inhibit the initiation and elongation reactions of RNA synthesis, respectively. Mutants which are resistant to these antibiotics have been shown to have altered $\beta$ subunits. In reconsti-

tution experiments, purified β subunit from a $rif^R$ mutant combined with the β' , σ and α subunits of a wild type RNA polymerase resulted in catalytically active enzyme which was not inhibited by rifampicin (73). Moreover, when β purified from wild type RNA polymerase was combined with the β' , σ and α subunits of RNA polymerase from a $rif^R$ mutant, the reconstituted enzyme was inhibited by rifampicin. Similar results have been obtained in experiments using streptolydigin-resistant mutants (74).

The α subunit is the smallest component of RNA polymerase (40 Kd). Tryptic peptide analysis of the two α subunits has demonstrated that they are identical polypeptides (75,76). The function of the α subunits has not been clearly demonstrated. The α subunit will not bind DNA and no catalytic activity is associated with this peptide by itself (9). Also, affinity, labeling experiments have not demonstrated any functional sites associated with the α subunits (9). However, no enzymatic activity can be detected when the α subunit is omitted from reconstitution experiments (62) and mutants which have an altered α subunit do not maintain transcriptional fidelity (77).

The sigma subunit (90 Kd) of RNA polymerase is responsible for the initiation of RNA synthesis at specific sites (promoters) on the DNA molecule, but is released shortly thereafter and is not necessary for the elongation or termination of RNA synthesis (63). The sigma subunit appears to be necessary for the formation of the first phosphodiester bond in the initiation of RNA synthesis and is thought to induce a conformational change in the core enzyme ($\beta\beta'\alpha_2$) which increases the binding affinity of RNA polymerase for DNA (64,65). Mutant analysis of the sigma subunit has also provided evidence that this polypeptide is involved in the regulation of transcriptional specificity (66). Purified RNA polymerase from these

mutants have altered sigma subunits and exhibit altered specificity in the transcription of bacterial and phage genes.

RNA polymerase mediates selective initiation of RNA synthesis by recognizing and binding to specific DNA sequences which are located 5' to the coding sequence of a gene (for reviews, see 9,78). These DNA sequences, or promoters, can be divided into two functional regions, each of which is necessary for the correct initiation of RNA synthesis in bacteria. The -35 region is located 30 to 36 base pairs upstream from the point of initiation. This region is A-T rich and is represented by the concensus sequence $\frac{\text{TTGACA}}{\text{AACTGT}}$ (79,80). Mutations in this region appear to prevent transcription *in vivo* (80) and DNA which is missing the -35 region is not used efficiently as a template *in vitro* (80).

The -10 region ("Pribnow box") of bacterial promoters is located 7-13 base pairs upstream from the start of transcription and is represented by the sequence $\frac{\text{TATPuATG}}{\text{ATAPyTAC}}$ (81,82). This DNA sequence is also A-T rich and appears to be crucial for the tight binding of RNA polymerase to DNA. RNA polymerase-DNA binding protection experiments have revealed that a DNA fragment containing 45 base pairs upstream and 20 base pairs downstream from the point of initiation is necessary for promoter recognition of RNA polymerase *in vitro* (83-85). Analysis of the 45 base pairs upstream from the transcriptional start site revealed the presence of consensus sequences in both the -35 and -10 region.

The method by which RNA polymerase recognizes specific promoter sequences in procaryotes is thought to occur by a two step process (9,78). Promoter regions are located by RNA polymerase through a series of random association-dissociation events on the DNA molecule. When the enzyme associates with a promoter sequence, a binary complex is formed (closed

promoter complex). In vitro, these complexes are characterized by their formation at low temperatures and their high sensitivity to heparin (86). This comlex is then converted into one in which the DNA strands have opened to allow the RNA polymerase molecule access to the template base pairs (open promoter complex). These complexes are highly stable, and are resistant to dissociation by polyanions or inactivation by rifampicin, in vitro (87). The formation of these complexes requires elevated temperatures and this has been attributed to an energy-dependent local melting of the DNA (9). It is the open promoter complex which is capable of initiating RNA synthesis and the process of initiation is governed by the concentration of open complexes and the concentration of the 5 -terminal nucleoside triphosphate (88).

The structural features of bacterial promoters which are involved in specific recognition by the RNA polymerase holoenzyme have been examined. UV-irradiation experiments in which RNA polymerase holoenzyme was cross-linked to promoter regions have shown that the $\sigma$ and $\beta$ subunits are covalently attached to the third base upstream (-3) and the second base downstream (+3), respectively, from the point of initiation of RNA synthesis (85,89). In other studies, the physical contacts made by RNA polymerase with the DNA sequence were compared using two different promoters, lac UV5 and T7 A3 (84,89,90). Both of these are strong promoters as characterized by their strong interactions and rapid association rates with RNA polymerase. In these experiments, the spatial order of bases which were in contact with RNA polymerase was similar even though the promoters differed in their nucleotide sequence. The polymerase enzyme appeared to interact exclusively with one face of the DNA molecule in the -35 region and with the back of the DNA molecule in the -10 region and

further downstream. These studies have been extended by comparing the base sequence in contact with RNA polymerase for 54 bacterial promoters and it was found that these sequences were highly conserved (91).

RNA polymerase in developmental systems

Selective transcription of genetic information during development can be accomplished through a variety of mechanisms. The observation that RNA polymerase can recognize specific base sequences in promoter regions led to the suggestion that regulatory proteins could replace the sigma polypeptide of the bacterial enzyme. The new or modified RNA polymerase would then be able to recognize and initiate transcription at new promoter sites (64). New or modified forms of RNA polymerase have been purified from a number of procaryotic developmental systems. In several cases, a novel form of RNA polymerase was shown to specifically recognize the promoters of developmentally regulated genes.

When E. coli is infected with T4, several new polypeptides are synthesized which bind specifically to RNA polymerase (92). These proteins are T4 gene products which appear to alter the transcriptional specificity of the enzyme during the course of the infection. The proteins, P12 and P22, are coded for by T4 genes 33 and 55, respectively, and are necessary for late gene expression in vivo (93). The P15 protein, the product of the alc gene, and the P10 protein may be involved in blocking host chromosome transcription (94,95).

In addition to these RNA polymerase-associated polypeptides, several well characterized modifications of RNA polymerase occur in T4-infected cells. Immediately after infection, one of the two $\alpha$ subunits is altered by the covalent attachment of adenosine and phosphate residues (96). This

modification is independent of phage protein synthesis and is coded for by the alt gene (97,98). Later in infection, both α subunits are modified by the transfer of an adenosine diphosphate ribose group to a specific arginine residue on the polypeptide (96,99). This modification is coded for by the product of the mod gene. While the significance of these modifications is not well understood, the modified holoenzyme is 4-10 times less capable of transcribing certain E. coli genes in vitro than RNA polymerase isolated from uninfected cells (10). This may indicate that T4 modification of E. coli RNA polymerase is involved in shutting-off transcription of host genes during the lytic cycle.

The Bacillus subtilis phage SP01 undergoes a well-defined developmental process during its lytic cycle. This temporal control of gene expression appears to be controlled at the level of promoter recognition (for review, see 10). The "early" genes of SP01 are transcribed by the unmodified host RNA polymerase soon after infection (100,101). Gene 28, one of these early genes, codes for a regulatory protein which functions as a sigma-like protein. This protein binds to the core RNA polymerase of B. subtilis and directs it to recognize "middle" gene promoters and initiate RNA synthesis (102,103). In a similar fashion, the protein products of middle genes 33 and 34 bind to the host core RNA polymerase later during infection and direct transcription of the SP01 genes expressed late in the lytic cycle (104).

This type of transcriptional control implies that the promoters of middle and late genes, which are recognized by these novel forms of RNA polymerase, are different in nucleotide sequence from those which are found for vegetative genes. The nucleotide sequences of several middle gene promoters have been analyzed and compared to the promoters of vegetative

genes (105,106). The nucleotide sequence for middle gene promoters was conserved in both the -35 and -10 regions. However, the sequence in these conserved regions was found to be dramatically different than that found in the -35 and -10 region of vegetative gene promoters. This would indicate that a similar mechanism of promoter selection may be used by the different forms of RNA polymerase in SP01-infected cells, but that selective transcription is achieved through the association of different sigma-like polypeptides with the core polymerase.

During the process of sporulation, B. subtilis also exhibits programmed gene expression (10). This developmental process can be correlated with the appearance of sigma-like regulatory proteins. These proteins bind to core RNA polymerase and alter its transcriptional specificity. Two such proteins, $\sigma^{37}$ and $\sigma^{29}$, have been purified (107,108). The first of these, $\sigma^{37}$, is present in vegetative cells and when associated with core RNA polymerase directs the transcription of two cloned sporulation genes, spo VG and spo VC, in vitro. Neither of these genes is recognized by the vegetatve holoenzyme which contains $\sigma^{55}$. Another sigma-like protein, $\sigma^{29}$, is synthesized during the early stages of sporulation. This protein can associate with core RNA polymerase and directs continued transcription of both the spo VG and spo VC genes. It also appears to direct transcription of at least one other sporulation specific gene in vitro. This type of transcriptional control may not be limited to sporulation specific genes in B. subtilis. An additional sigma-like protein, $\sigma^{28}$, has been discovered which is present in vegetative cells (109). When associated with core RNA polymerase, this enzyme utilizes a distinct promoter on T7 DNA, not recognized by the normal vegetative holoenzyme.

The promoter sequences recognized by these alternate forms of B.

*subtilis* RNA polymerase have been examined (110-112). Again, as with SP01 promoters, the -35 and -10 regions of genes transcribed by similar forms of the holoenzyme appear to be conserved. However, in the case of both $\sigma^{37}$ and $\sigma^{29}$, there appear to be critical differences in the DNA sequence in these regions when compared with promoters which are recognized by alternate forms of the enzyme.

# MATERIALS AND METHODS

## Materials

Guanosine triphosphate (GTP), cytidine triphosphate (CTP), uridine triphosphate (UTP) and calf thymus DNA were purchased from P-L Biochemicals, Milwaukee, WI. [5,6-$^{3}$H]UTP (30 Ci/mmol) and [U-$^{14}$C]ATP (58 mCi/mmol) were purchased from ICN, Irvine, CA. [$\alpha$-$^{32}$P]ATP and POP were purchased from New England Nuclear, Boston, MA. Adenosine triphosphate (ATP), lysozyme, rifampicin, EtBr, polyvinyl pyrrolidone, Dowex-50 and heparin were purchased from Sigma Chemical Co., St. Louis, MO. Cellulose, DEAE-cellulose, 3MM paper and GF/C glass filters were purchased from Whatman, Clinton, NJ. Nitrocellulose (BA84, 0.45 $\mu$m) was purchased from Schleicher and Schuell, Keene, NH. Agarose (standard low-$m_r$) and SDS were purchased from Bio-Rad Laboratories, Richmond, CA. DNase I (DPFF) was purchased from Worthington Biochemicals, Freehold, NJ. RNasin was purchased from Bio Tech, Madison, WI. All restriction enzymes with the exception of Eco RI were purchased from Bethesda Research Laboratories, Inc., Gaitersburg, MD. Eco RI was a gift from Dr. A. Revzin, Michigan State University. Triton-X 100 and dimethyl-POPOP were purchased from Research Products International Corp., Mount Prospect, IL. Yeast extract, Bacto-agar, Bacto-tryptone and casamino acids were purchased from Difco Laboratories, Detroit, MI. X-ray film (XAR-5) was purchased from Kodak,

Rochester, NY. All other chemicals were reagent grade and were purchased from standard sources.

The bacterial strains, plasmids and phage used are listed in Table II.

Media and buffers

T medium - Tryptone (T) broth contained (per liter): 10 g Bacto-tryptone, 5 g NaCl (115).

YM medium - Yeast extract-mannitol (YM) broth contained (per liter): 10 g mannitol, 0.2 g $KH_2PO_4$, 0.3 g $K_2HPO_4$, 0.2 g $MgSO_4$ $^7H_2O$, 0.1 g NaCl, 2.0 g yeast extract (116).

LB medium - Luria-Bertani (LB) broth contained (per liter): 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl and 0.1% (w/v) glucose (115).

M9 medium - M9 broth contained (per liter): 6 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g $NH_4Cl$, 2.4 g $MgSO_4$, 0.01 g $CaCl_2$, 0.2% (w/v) glucose (115).

M9CA medium - M9CA medium consisted of M9 medium + 1% (w/v) casamino acids (115).

Modified TGED buffer - Modified TGED buffer consisted on 10 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 25% (v/v) glycerol.

TB buffer - Tris-borate (TB) buffer consisted of 89 mM Trizma base, 8.9 mM EDTA and 89 mM boric acid (DNA gel electrophoresis buffer) (115).

TE buffer - Tris-EDTA (TE) buffer consisted of 10 mM Tris, pH 8.0, 1 mM EDTA (115).

RNA gel electrophoresis buffer - RNA gel electrophoresis buffer consisted of 40 mM Tris, pH 7.4, 20 mM sodium acetate, 1 mM EDTA (117).

SDS-polyacrylamide gel electrophoresis buffer - SDS-polyacrylamide gel electrophoresis buffer contained (per liter): 28.75 g glycine, 1 g SDS, 6 g

Table II. Bacterial strains, plasmids and phage

| Strains | Description | Source |
|---|---|---|
| *R. japonicum* 3I1b 110 | w.t., symbiotically effective | D. Weber, Beltsville, MD |
| *E. coli* HB101 (pRJ676) | $F^-$, *rec* A13, ($Amp^r$) | H. Hennecke (110) |
| *E. coli* HB101 (pRJ676 1) | $F^-$, *rec* A13, ($Amp^r$) | B. Chelm, MSU |
| *E. coli* HB101 (pSA30) | $F^-$, *rec* A13, ($Tet^r$) | F.M. Ausubel (114) |
| *E. coli* HB101 (pRmR2) | $F^-$, *rec* A13, ($Tet^r$) | F.M. Ausubel (57) |
| *E. coli* D10 | $F^-$, RNase $I^-$, *met*, *thi* (T7 host) | S. Shamblatt, MSU |
| *E. coli* K803 | *met*, *gal*, am $su^+$ (T4 host) | L. Snyder, MSU |
| **Plasmids** | | |
| pRJ676 | contains *R. japonicum* *nif* genes, $Amp^r$ | see above |
| pRJ676 1 | contains *R. japonicum* *nif* genes, $Amp^r$ | see above |
| pSA30 | contains *K. pneumoniae* *nif* genes, $Tet^r$ | see above |
| pRmR2 | contains *R. meliloti* *nif* genes, $Tet^r$ | see above |
| **Phage** | | |
| T7 | w.t. | S. Shamblatt, MSU |
| T7 D111 | deletion of $A_2$ and $A_3$ promoters | S. Shamblatt, MSU |
| T4 | w.t. | L. Snyder, MSU |

Trizma base (118).

SSC buffer - Saline-sodium citrate (SSC) buffer consisted of 0.15 M NaCl, 0.015 M sodium citrate (115).

SDS-polyacrylamide gel sample buffer - SDS-polyacrylamide gel sample buffer consisted of 0.12 M Tris, pH 6.8, 2.5% (w/v) SDS, 1.2 M β-mercaptoethanol, 20% (v/v) glycerol, 0.1% (w/v) bromphenol blue (118).

RNA gel sample buffer - RNA gel sample buffer consisted of 0.1 M aurintricarboxylic acid, 20 mM EDTA, 0.1% (w/v) bromphenol blue, 60% (v/v) glycerol (117).

DNA gel sample buffer - DNA gel sample buffer consisted of 89 mM Trizma base, 8.9 mM EDTA, 89 mM boric acid, 60% (v/v) glycerol, 0.1% (w/v) bromphenol blue (115).

Phage buffer A - Phage buffer A consisted of 10 mM Tris, pH 7.9, 1mM EDTA, 1 M NaCl and 0.01% (w/v) gelatin.

## Preparation of RNase-free solutions and glassware

All glassware and metal spatulas were washed in 50% methanol, 4 M potassium hydroxide overnight, rinsed with warm water, dried and baked at 250 C for a minimum of 8 h. All solutions which did not contain a primary amine were placed in RNase-free glassware and DEPC (50 µl/liter of solution) was added. These solutions were then autoclaved before use. All solutions containing a primary amine were made with RNase-free $H_2O$ in RNase-free glassware and chemicals were transferred with baked spatulas. Eppendorf tubes were autoclaved and dried at 60 C before use.

## Growth of R. japonicum 110

R. japonicum strain 3I1b 110 used was used in all studies and will be referred to as R. japonicum 110. Bacteria were grown on a gyratory shaker (200 rpm) at 30 C in YM media. Cultures of cells (100 ml) used for growth curve measurements were inoculated from a single colony and were grown in 500-ml side-arm flasks. Growth was monitored at the indicated times using a Klett colorimetor with a filter which measured in the range of 500 to 570 mμ. Cultures of cells (1 liter) used for RNA polymerase purification were grown in 2800-ml Fernbach flasks. The cultures were inoculated with 10 ml of a 3-day-old culture and were harvested after 6 days by centrifugation for 10 min at 3000 x g. Harvested cells were either used immediately or stored at -20 C for up to 2 months.

## Plant growth

Soybean seeds (Glycine max c.v. Amsoy 71) were inoculated with liquid cultures of R. japonicum 110 and planted in 20-cm pots filled with Perlite. The plants were maintained on N-free nutrient solution (119) with a 16 h photoperiod. The lighting conditions of the greenhouse were 200 μE/m/s at pot level. Nodules were harvested from 40 to 50-day-old plants and stored at -20 C.

## Isolation of bacteroids

Frozen nodules (100 g) were suspended in 200 ml of a buffer containing 25 mM potassium phosphate, 200 mM ascorbate, pH 7.2 and 33 g of acid washed polyvinyl pyrrolidone. The nodules were homogenized in a Waring blender at high speed for 1-2 min at 4 C. The homogenate was filtered through 3 layers of cheesecloth followed by 1 layer of Miracloth. The filtrate was

centrifuged at 3000 x g for 5 min at 4 C. The upper part of the pellet containing the bacteroids was resuspended and washed in 100 mM Tris, pH 7.9 and centrifuged at 3000 x g for 5 min at 4 C. The pellet was used immediately or stored at -20 C.

Glycerol gradient ultracentrifugation

RNA polymerase was applied to a 5-ml linear gradient of 10-30% glycerol containing 10 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10 mM $MgCl_2$ and 1 M KCl. The gradients were centrifuged at 25,000 rpm in a SW 50.1 rotor for 24 h at 4 C and the fractions were collected and assayed immediately for RNA polymerase activity using the standard RNA polymerase assay. Purified β-galactosidase and catalase, which were added to the RNA polymerase samples prior to ultracentrifugation, were assayed according to standard procedures (120,121).

Protein determination

Protein concentration was determined by the method of Lowry, et al. (122). Bovine serum albumin was used as a standard.

SDS-polyacrylamide gel electrophoresis

Protein samples were analyzed by gel electrophoresis on 8.75% polyacrylamide slab gels (1.5 mM thick) containing 0.1% (w/v) SDS according to the procedure of Laemmli and Favre (118). SDS-sample buffer was added to the protein samples and these were heated at 100 C for 2 min. Samples were subjected to electrophoresis at 40 mA for 3-4 h. The gels were fixed in 10% TCA for 1 h and stained overnight in 0.125% Coomassie Blue R:25% isopropanol:10% acetic acid (w/v/v). Gels were destained in 10% acetic

acid.

Purification of T7 DNA

T7 DNA was purified using the procedure of Minkley and Pribnow (123). Liter cultures of E. coli D10 were grown in T-broth in 2800-ml Fernbach flasks on a gyratory shaker at 30 C to a cell density of 6 x $10^8$ cells/ml. Cultures were then infected with T7 phage at a multiplicity of 0.1 and were shaken at 30 C until complete lysis had occurred. Solid NaCl was added to the cultures to a final concentration of 2.1% (w/v) and the solution was stirred at 4 C for 10 min. Solid PEG 6000 was added to the solution to a final concentration of 10% (w/v) and the solution was stirred at 4 C for at least 2 h. The solution was then centrifuged at 6000 x g for 5 min and the pellet was resuspended in phage buffer A (20 ml/l of cells). This solution was centrifuged at 6000 x g for 15 min and the pellet was washed three times by a series of centrifugations and resuspensions in 10 ml of phage buffer A. All supernatant fluids were combined and the T7 phage were pelleted by centrifugation at 30,000 rpm for 90 min in a Ti50 rotor. The phage pellet was resuspended in phage buffer A (1.5 ml/l of lysate) and clarified by centrifugation at 10,000 x g for 10 min. The supernatant fluid was collected and the T7 phage were further purified using 5-ml CsCl step gradients. CsCl solutions were prepared in 10 mM Tris, pH 7.9, 0.1 mM EDTA. Gradients were composed of 0.5 ml of 62.5% (w/w) CsCl, 1.0 ml of 41.7% (w/w) CsCl, 1.0 ml of 31.3% (w/w) CsCl and 1.0 ml of 20.8% (w/w) CsCl. The T7 phage stock (1.5 ml) was layered on top of the gradient and the gradients were centrifuged at 35,000 rpm for 1 h in a SW 50.1 rotor. The gradients were fractionated and the visible bands containing the T7 phage were dialyzed for 2 days against 4 x 1 liter of 10 mM Tris, pH 7.9,

10 mM $MgCl_2$, 0.1 M NaCl. T7 phage were diluted with dialysis buffer until the absorbance at 260 nm was less than 10 and SDS was added to a final concentration of 0.5% (w/v). This solution was incubated at 55 C for 15 min after which KCl was added to a final concentration of 0.4 M. The solution was chilled for 15 min on ice and centrifuged at 10,000 x g for 15 min. The supernatant fluid was phenol extracted and dialyzed for 2 days against 6 x 1 liter of 10 mM Tris, pH 7.9, 0.1 M NaCl, 0.5 mM EDTA and the T7 DNA was stored at 4 C.

Purification of T4 DNA

M9CA broth (50 ml) was inoculated with 2 ml of an overnight culture of E. coli K803. The culture was grown at 30 C for 3 h and was then used to inoculate 500 ml of M9CA broth in a 2800-ml Fernbach flask. The culture was grown at 30 C for 30 min, inoculated with 3 x $10^9$ phage in 0.1 ml and grown for an additional 6 h at 30 C. To ensure complete lysis, 10 ml of chloroform was then added to the culture and cellular debris was removed by centrifugation at 5000 x g for 10 min at 4 C. The T4 phage were harvested by centrifuging the supernatant fluid at 27,000 x g for 1 h. The pellet was resuspended in 5 ml of M9CA broth and this solution was clarified by centrifugation at 5000 x g for 10 min. The supernatant fluid was collected and T4 phage were further purified using 5-ml CsCl step gradients. CsCl solutions were prepared in re-distilled $H_2O$. Gradients were composed of 0.5 ml each of (w/v) 65%, 60%, 50%, 40%, 30%, and 20% CsCl solutions. The T4 phage stock (1.5 ml) was layered on top of the gradient and the gradients were centrifuged at 35,000 rpm in a SW 50.1 rotor for 20 min. The gradients were fractionated and the visible bands containing T4 phage were dialyzed against 1 liter of 2 M, 1 M, 0.5 M NaCl for 2 h each and then

against 1 liter of M9CA media for 24 h. T4 DNA was purified by phenol extraction and the DNA was dialyzed against 2 x 1 liter of 10 mM Tris, pH 7.5, 0.15 M NaCl for 24 h. T4 DNA was stored at 4 C.

Plasmid purification

Plasmid DNA was purified essentially as described by Clewell and Helinski (124). A 10-ml culture of LB or M9 media containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated at 37 C overnight with vigorous shaking. This culture was used to inoculate 1 liter of broth in a 2800-ml Fernbach flask. The culture was incubated at 37 C with shaking until the cell density was equal to $4 \times 10^8$ cells/ml ($OD_{550}$=0.5 is equivalent to $5 \times 10^8$ cells/ml) after which solid chloramphenicol (150 mg) was added. The culture was then incubated at 37 C with shaking for 16-18 h. The bacterial cells were harvested by centrifugation at 4000 x g for 10 min at 4 C. The cells were washed with 20 ml of 10 mM Tris, pH 8.0, 25% (w/v) sucrose and then resuspended in 50 ml of this buffer. Lysozyme (7 ml of a 5 mg/ml solution in 0.25 M Tris, pH 8.0) was added to the cells and the solution was incubated on ice for 5 min after which 15 ml of 0.25 M EDTA, pH 8.0 was added. The cells were incubated on ice for 15 min followed by the addition of 8 ml of 10% (w/v) SDS. The solution was mixed gently and 20 ml of 5 M NaCl was added, bringing the final NaCl concentration to 1 M. The solution was then incubated on ice for 90 min and centrifuged at 30,000 x g for 30 min at 4 C. The supernatant fluid was extracted with an equal volume of phenol and then re-extracted with an equal volume of chloroform. The aqueous phase was removed, 0.1 volumes of 3 M sodium acetate, pH 8.0 and 2 volumes of 95% ethanol were added and plasmid DNA was precipitated by incubation

overnight at -20 C followed by centrifugation at 12,000 x g for 20 min at 4 C. The DNA pellet was air-dired and resuspended in 100 µl of TE buffer. Plasmid DNA was further purified by EtBr-CsCl density gradient centrifugation. Solid CsCl (10 g) was added to 10 ml of 80 mM Tris, pH 8.0, 20 mM EDTA containing 300 to 500 µg of ethanol precipitated DNA. Ethidium bromide (200 µg) was added to the CsCl solution and the gradients were centrifuged for 24 h at 35,000 rpm in a Ti 50 rotor. The plasmid band was removed from the gradients and the DNA was dialyzed against 4 x 1 liter of TE buffer. Ethidium bromide was removed from the plasmid DNA by Dowex-50 chromatography in TE buffer and the DNA was concentrated by ethanol precipitation at -20 C overnight. Contaminating RNA was removed from plasmid DNA by Agarose A 1.5 M chromatography in TE buffer, and if necessary, DNA was again concentrated by ethanol precipitation. Fractions from Dowex-50 and Agarose A 1.5 M columns were monitored at an absorbance of 260 nm and assayed by agarose gel electrophoresis.

Standard RNA polymerase assay

RNA polymerase was assayed in a mixture consisting of (final volume, 100 µl): 10 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 mM ATP, 0.15 mM CTP, 0.15 mM GTP, 0.038 mM UTP, 2.5 µCi [$^3$H]UTP, 10 mM MgCl; 15 µg calf thymus DNA, 15 µg BSA. The reaction was initiated by the addition of enzyme, incubated at 37 C for 10 min, and stopped by the addition of 100 µl of 1% (w/v) SDS, 10 mM EDTA. Two ml of cold 10% (w/v) TCA were added to each sample and the reaction vials were kept on ice for 30 min. Each sample was then filtered onto Whatman GF/C filters and washed with 10 ml of cold 10% TCA. The filters were dried and the amount of radioactivity present was determined by liquid scintillation spectrometry. The scin-

tillation fluid used consisted of (per liter): 333 ml Triton X-100, 666 ml toluene, 5 g PPO and 0.1 g POPOP.

Inhibition of in vitro RNA synthesis by rifampicin and heparin

RNA polymerase (1 μg) and the indicated amount of rifampicin or heparin were added to the standard reaction mixture lacking the nucleoside triphosphates. The reaction was started by the addition of the nucleoside triphosphates, incubated at 37 C for 10 min, and stopped by the addition of 100 μl of 1% (w/v) SDS, 10 mM EDTA. The samples were then treated in a manner similar to that described in the standard RNA polymerase assay.

T4 RNA polymerase assay

RNA polymerase was assayed in a manner similar to that described for the standard RNA polymerase assay except that 6 μg of T4 DNA was substituted for calf thymus DNA. Samples were incubated at 37 C for 20 min and the reaction was stopped with the addition of 100 μl of a solution containing 50 mM sodium pyrophosphate, 50 mM EDTA and 0.5 mg/ml tRNA. Samples were then treated as described for the standard RNA polymerase assay.

T7 RNA polymerase kinetic assays

RNA polymerase assays used to determine the elongation rate and termination efficiency of RNA polymerase were carried out according to the method of Chamberlin et al. (11). The reaction mixture contained 0.4 ml of AB diluent (10 mM Tris, pH 8.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 mM NaCl, 0.1 mM EDTA, 5% (v/v) glycerol, 100 μg/ml BSA), 0.2 ml of solution A (0.2 M Tris, pH 8.0, 50 mM $MgCl_2$, 50 mM β-mercaptoethanol), 12.5 μg T7 DNA, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, and 0.6

μCi [$^{14}$C]ATP in a final volume of 1 ml. A sample (100 μl) was removed as a zero time point and was mixed with 200 μl of carrier solution (50 mM sodium pyrophosphate, 50 mM EDTA, 0.5 mg/ml yeast tRNA). Cold 10% TCA (2.5 ml) was added to precipitate nucleic acids and the sample was placed on ice. RNA polymerase (3 μg) was added to the reaction mixture and RNA synthesis was initiated by transferring the tube to a water bath at 30 C. At 1.8 min, 10 μl of heparin (10 mg/ml) was added to the reaction. Samples (100 μl) were taken at appropriate time intervals, mixed with 200 μl of carrier and nucleic acids were precipitated with 2.5 ml of cold 10% TCA. The samples were placed on ice for 30 min and were then filtered onto Whatman GF/C filters. Each filter was washed with 35 ml of cold 1 M HCl, 0.1 M sodium pyrophosphate, followed by 10 ml of cold 95% ethanol. The filters were dried and the amount of radioactivity present was determined by liquid scintillation spectrometry.

Analysis of T7 promoters utilized by RNA polymerase in vitro

To determine the specificity of promoter utilization of RNA polymerase from R. japonicum, $^{32}$P-labeled RNAs were synthesized under the prebinding conditions of Wiggs et al. (125). RNA polymerase from vegetative cells (2 μg), or bacteroids (2 μg), or from E. coli (1 μg) was incubated at 37 C for 5 min with 2.4 μg of T7 DNA in a reaction mixture lacking nucleoside triphosphates (125). The reaction was started by the addition of the nucleoside triphosphates and after 5 min, 10 μl (10 μg) of heparin was added to prevent reinitiation of transcription during the incubation. After 20 min, the reaction was stopped by the addition of 25 μl of a solution consisting of 0.1 M EDTA, 0.1% (w/v) SDS, 27 mM boric acid and 30% (v/v) glycerol and the samples were placed on ice. A 10 μl sample of the

reaction mixture was assayed for incorporation of [$^{32}$P]UMP into acid-insoluble material. The RNA synthesized *in vitro* was analyzed by agarose-acrylamide gel electrophoresis according to the method of Golomb and Chamberlin (126). RNA samples containing 30,000-50,000 cpms were heated in a boiling $H_2O$ bath for 30 sec and rapidly cooled to 0 C. The RNA samples were then applied to a 25 cm x 0.2 cm vertical agarose-acrylamide gel and subjected to electrophoresis at 40 V for approximately 16 h. The gel was incubated in EtBr (5 µg/ml) for 15 min and photographed using a UV trans-illuminator. The gel was then dried onto 3MM paper, wrapped in Saran wrap and analyzed by autoradiography using Kodak XAR-5 film at -80 C with an intensifying screen.

Ternary transcription complex analysis

The ability of RNA polymerase to recognize *nif* promoters on pRJ676 (113), a recombinant plasmid containing the *nif* DK genes of *R. japonicum*, was tested using the pre-cut procedure of Chelm and Geiduschek (117). Plasmid DNA was restricted with Eco R1 and Hind III using standard conditions for Hind III (Bethesda Research Laboratories catalogue) and 2 units of enzyme per µg of DNA. The reaction was terminated by heating the reaction mixture at 65 C for 10 min. RNA polymerase was then assayed in a mixture consisting of (final volume, 30 µl): 10 mM Tris, pH 8.0, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.8 mM spermidine, 50 mM NaCl, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 5 µM UTP, 3 µCi [$\alpha$-$^{32}$P]UTP, and 1 µg restricted pRJ676 DNA. RNA polymerase from vegetative cells or bacteroids was incubated at 37 C for 5 min in the reaction mixture lacking nucleoside triphosphates. The reaction was started by the addition of nucleoside triphosphates and was stopped after 30 sec by the addition of 10 µl

of a solution consisting of 40% (v/v) glycerol, 80 mM EDTA, 0.2 mM aurintricarboxylic acid and 0.08% (w/v) bromphenol blue. A 5 µl sample of the reaction mixture was assayed for incorporation of [$^{32}$P]UMP into acid-insoluble material while the remainder of the sample was analyzed by agarose gel electrophoresis (117). Ternary transcription complexes were applied to a 25 cm x 0.2 cm vertical gel agarose gel and subjected to electrophoresis at 50 V overnight. The gel was incubated in EtBr (0.5 µg/ml) for 15 min and photographed using a UV transilluminator. The gel was then dried onto 3MM paper, wrapped in Saran wrap and analyzed by autoradiography using Kodak XAR-5 film at -80 C with an intensifying screen.

RNA synthesis for southern hybridizations

RNA was synthesized *in vitro* in a mixture consisting of (final volume, 100 µl): 10 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 mM ATP, 0.15 mM GTP, 0.15 mM CTP, 3 µM UTP, 3 µCi [$\alpha$-$^{32}$P]UTP, 10 mM MgCl, 15 µg BSA, 3 µg plasmid DNA. RNA polymerase from vegetative cells (6 µg), or bacteroids (6 µg) or from *E. coli* (2 µg) was preincubated at 37 C for 5 min in this reaction mixture lacking the nucleoside triphosphates. The reaction was started by the addition of the nucleoside triphosphates and was stopped after 6 min by placing the reaction vials on ice and adding 0.1 volumes of 3 M sodium acetate and 2 volumes of 95% ethanol. Yeast tRNA (100 µg) and single stranded DNA (100 µg) were added as carrier and the $^{32}$P-labeled RNA was ethanol precipitated overnight at -20 C followed by centrifugation at 10,000 x g for 10 min at 4 C. The pellet was dried, resuspended in 100 µl of TE buffer and a 1 µl aliquot was assayed for incorporation of [$^{32}$P]UMP into acid-insoluble material. The $^{32}$P-labeled RNA was then used in southern hybridizations to restricted plasmid DNA (108).

Restriction of plasmid DNA

Plasmid DNA was restricted with the appropriate restriction enzymes using standard conditions (Bethesda Research Laboratories catalogue) and 2 units of enzyme per μg of DNA. The reactions were terminated by incubating the reation mixture at 65 C for 10 min.

Southern transfers and hybridizations

Restricted DNA fragments were separated by agarose gel electrophoresis (127) and transfer of DNA from agarose gels to nitrocellulose filters was performed according to the method of Southern (128). Transfer of the DNA was allowed to proceed for 48 h using 6 x SSC buffer after which the nitrocellulose filters were removed, dried, placed between 2 sheets of 3 MM paper and baked for 2 h at 80 C under vacuum. Nitrocellulose strips containing immobilized DNA fragments were wet with hybridization buffer (5 x SSC, pH 7.4, 50% (v/v) formimide, 300 μg/ml yeast tRNA, 100 μg/ml single stranded DNA) and were pre-annealed in 10 ml of hybridization buffer for 6 h at 37 C. Radioactive RNA was added to the filters (approximately 200,000 cpms/filter) and the reaction mixtures were incubated with gentle shaking at 37 C for 18 h. The filters were washed with hybridization buffer at 37 C for 90 min followed by 2 consecutive 15 min washes with 2 x SSC at room temperature. The nitrocellulose filters were then incubated with pancreatic RNase (20 μg/ml) at room temperature for 1 h. The filters were flattened, dried at 80 C for 2 h under vacuum and Kodak XAR-5 film was exposed at -80 C with an intensifying screen.

# CHAPTER I

## PURIFICATION AND PHYSICAL CHARACTERIZATION OF RNA POLYMERASE FROM VEGETATIVE CELLS AND BACTEROIDS OF *RHIZOBIUM JAPONICUM*

During the development of the *Rhizobium*-legume symbiosis, the induction or depression of a number of bacterial gene products is necessary for the process of biological nitrogen fixation. However, the transcriptional control mechanism(s) which is responsible for this selective gene expression is, as yet, unknown. As a first step in analyzing the transcriptional control of the *nif* and symbiotic genes *in vitro*, RNA polymerase was purified from vegetative cells and bacteroids of *R. japonicum* 110. In Chapter I, the purification of RNA polymerase from both vegetative cells and bacteroids of *R. japonicum* is presented as well as a physical characterization of the enzyme from both sources. The subunit composition of purified RNA polymerase from both forms of *R. japonicum* is compared and a sigma-like protein is identified, using procedures developed for the isolation of the sigma factor from *E. coli* RNA polymerase.

# RESULTS

## Growth of vegetative cells

*R. japonicum* 110, a slow-growing species of *Rhizobium*, was used for the purification of RNA polymerase. A growth curve of *R. japonicum* 110 is presented in Figure 2. The generation time of vegetative cells during the exponential phase of growth was 12-14 h. RNA polymerase activity was measured in the crude extracts of cells harvested at different times during the growth period (Table III). Total RNA polymerase activity, as measured by the standard *in vitro* assay, was highest in the crude extracts of cells after 5-6 days of growth. Therefore, vegetative cells used for the purification of RNA polymerase were harvested after 5 days of growth at 30 C. This corresponded to the late exponential-early stationary phase of growth.

## Purification of RNA polymerase holoenzyme

RNA polymerase was purified from vegetative cells and bacteroids of *R. japonicum* 110 by the method of Gross *et al.* (129) with the following modifications. All steps of the purification were carried out at 4 C. Freshly harvested or frozen cells (20 g wet weight) were suspended in 20 ml of lysis buffer (for bacteria: 100 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol; for bacteroids: 150 mM Tris, pH 8.8, 0.1 mM EDTA, 0.1 mM dithiothreitol) and were disrupted by passage through a French press (1-2 passes, 16,000 psi). The crude extract was centrifuged at 27,000 x g for 20 min. Solid NaCl and PEG 6000 were added to the supernatant fluid to a final concentration of 0.3 M NaCl, 10% PEG 6000 (w/v). This solution was stirred for approximately 40 min and centrifuged at 5900 x g for 10 min. The pellet was suspended in 20 ml of a solution containing 10 mM Tris,

Figure 2 - Growth curve of R. japonicum 110. A single colony of R. japonicum 110 was used to inoculate 100 ml of YM broth in a 500-ml side-arm flask. The culture was grown as described in Materials and Methods and growth was monitored at the indicated time points using a Klett colorimeter.

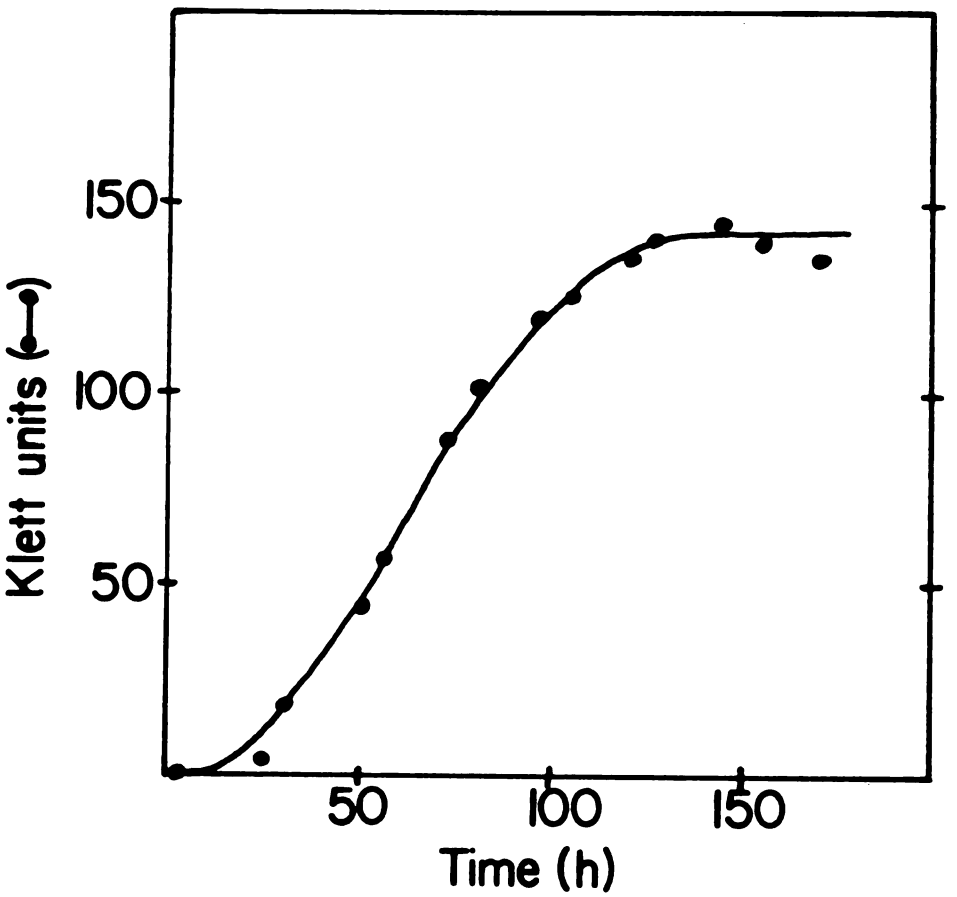



Figure 2

Table III. RNA polymerase activity in crude extracts of *R. japonicum* 110

| Culture Age[a] (days) | Total Protein[b] (mg) | Total Activity[c] (U) | Specific Activity (U/mg) |
|---|---|---|---|
| 2 | 0.5 | 1.1 | 2.2 |
| 3 | 0.95 | 3.7 | 3.9 |
| 4 | 1.5 | 3.9 | 2.6 |
| 6 | 3.6 | 9.8 | 2.7 |

(a) cells were harvested by centrifugation at 5900 x g for 10 min and were resuspended in 5 ml of 10 mM Tris, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol.

(b) protein was determined by the method of Lowry *et al.* (122). Bovine serum albumn was used as a standard.

(c) one unit of RNA polymerase activity is defined as the incorporation of one nmol of UMP into TCA-precipitable material in 10 min at 37 C.

pH 7.9, 10 mM dithiothreitol, 5% PEG 6000 (w/v), 2 M NaCl and stirred for approximately 30 min or until the pellet was completely in solution. The supernatant fluid was diluted with Modified TGED until the final NaCl concentration was equal to 0.15 M and was applied to a DNA-cellulose column (3.5 x 4.5 cm). The column was washed with 100 ml of Modified TGED + 0.15 M NaCl and the enzyme was eluted with a 200-ml linear gradient from 0.15 M to 1.0 M NaCl in Modified TGED. The column fractions were monitored at 280 nm and assayed for RNA polymerase activity using the standard RNA polymerase assay. RNA polymerase activity from both vegetative bacteria (Figure 3) and bacteroids (Figure 4) was eluted from the DNA-cellulose column at 0.55 M NaCl. The majority of RNA polymerase activity from both bacteria and bacteroids was consistently eluted as a double peak from the DNA-cellulose column. However, when these fractions were analyzed by SDS-polyacrylamide gel electrophoresis, no major difference in protein banding pattern could be detected.

Fractions from the DNA-cellulose column containing RNA polymerase activity were pooled, diluted with Modified TGED until the final NaCl concentration was equal to 0.1 M, and applied to a DEAE-cellulose column (2.0 x 6.5 cm). The column was washed with 50 ml of Modified TGED + 0.1 M NaCl and the enzyme was eluted with a 100-ml linear gradient from 0.1 M to 1.0 M NaCl in Modified TGED. RNA polymerase activity was eluted from the DEAE-cellulose column at 0.2 M NaCl from both vegetative bacteria (Figure 5) and bacteroids (Figure 6). Fractions which contained RNA polymerase activity were collected and stored at -20 C.

A summary of the purification of RNA polymerase from vegetative cells and bacteroids of R. japonicum 110 is presented in Table IV. Aside from differences in the total and specific activities in crude extracts, the

Figure 3 - Elution profile of RNA polymerase from vegetative cells of R. japonicum 110 after DNA-cellulose chromatography. The 2 M NaCl supernatant was diluted with Modified TGED, applied to a column (3.5 x 4.5 cm) of DNA-cellulose and RNA polymerase was eluted as described in the text. Fractions (2 ml) were collected and monitored for absorbance at 280 nm (●—●). RNA polymerase activity (□—□) was determined by assaying 10 µl samples of the column fractions. NaCl concentrations (-----) were determined by conductivity measurements.

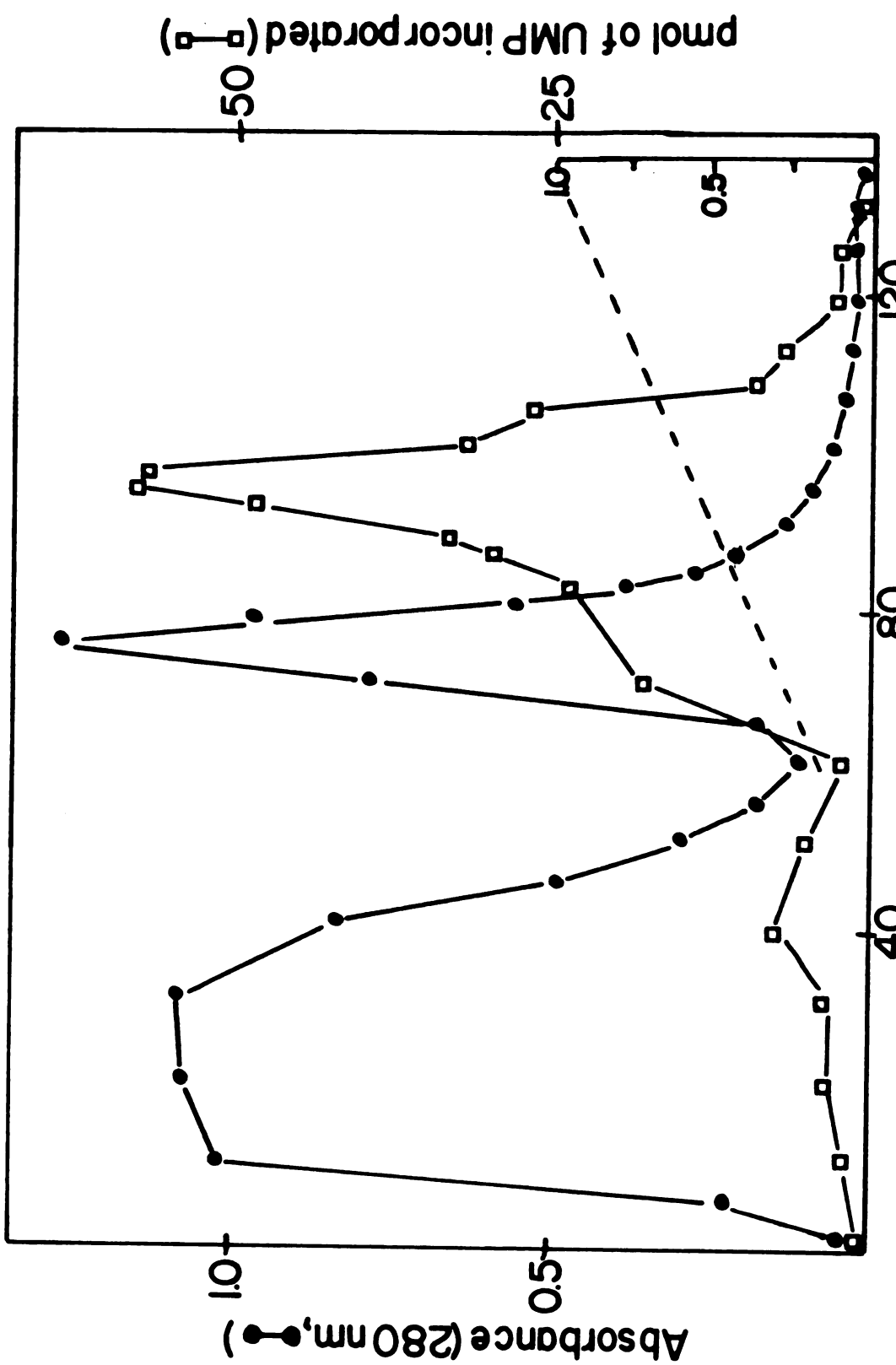

Absorbance (280 nm, ●—●)
1.0
0.5
pmol of UMP incorporated (□—□)
50
25
10
0.5
40
80
120
Fraction number

Figure 3

Figure 4 - Elution profile of RNA polymerase from bacteroids of *R. japonicum* 110 after DNA-cellulose chromatography. The 2 M NaCl supernatant was diluted with Modified TGED, applied to a column (3.5 x 4.5 cm) of DNA-cellulose and RNA polymerase was eluted as described in the text. Fractions (2 ml) were collected and monitored for absorbance at 280 nm (●—●). RNA polymerase activity (□—□) was determined by assaying 10 µl samples of the column fractions. NaCl concentrations (----) were determined by conductivity measurements.

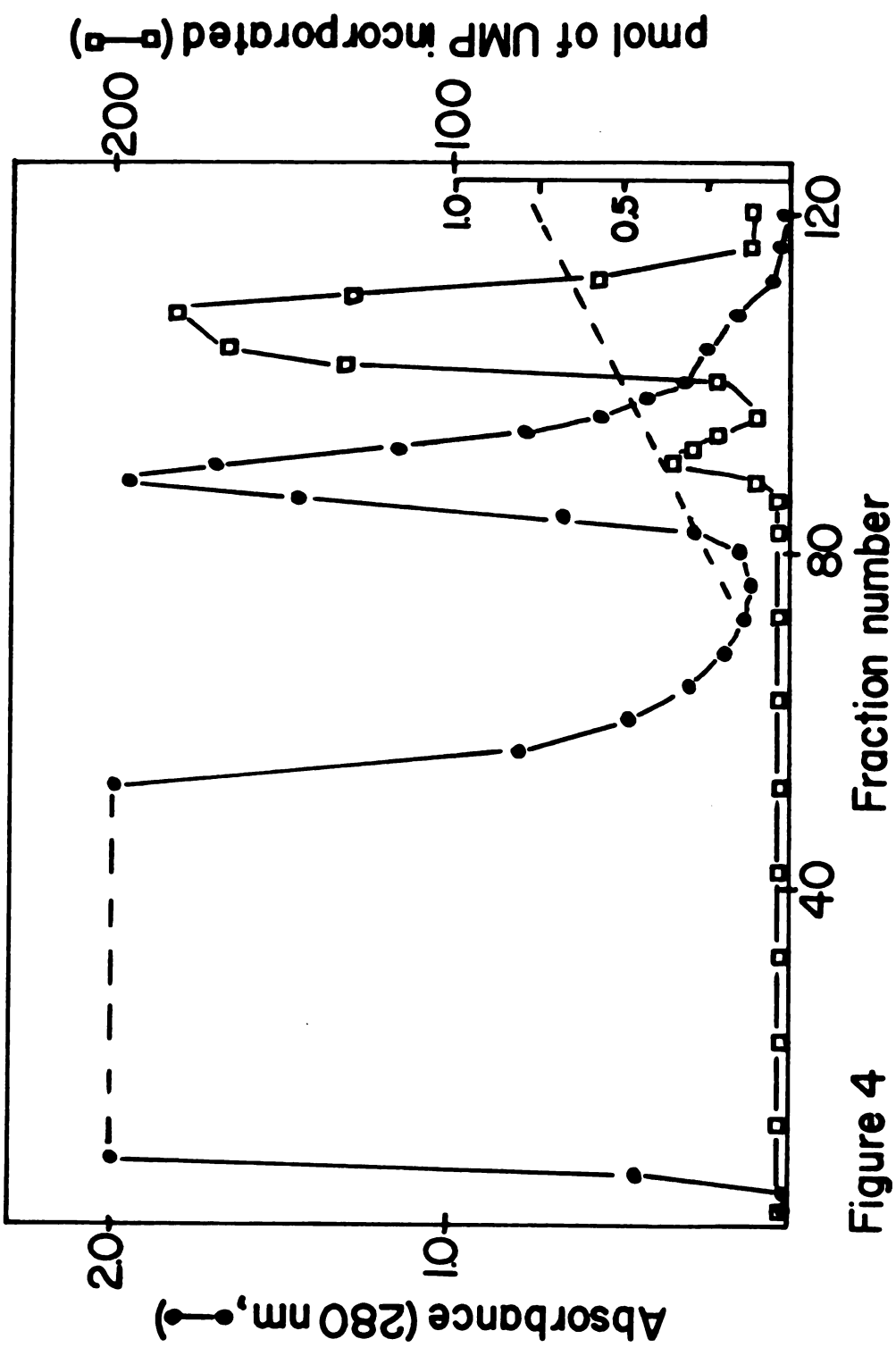

Absorbance (280 nm, ●—●)
20
10
pmol of UMP incorporated (□—□)
200
100
1.0
0.5
40
80
120
Fraction number

Figure 4

Figure 5 - Elution profile of RNA polymerase from vegetative cells of R. japonicum 110 after DEAE-cellulose chromatography. Fractions (85-115) from the DNA-cellulose column were diluted with Modified TGED, applied to a column (2.0 x 6.5 cm) of DEAE-cellulose and RNA polymerase activity was eluted as described in the text. Fractions (2 ml) were collected and monitored for absorbance at 280 nm (●—●). RNA polymerase activity (□—□) was determined by assaying 10 µl samples of the column fractions. NaCl concentrations (----) were determined by conductivity measurements.

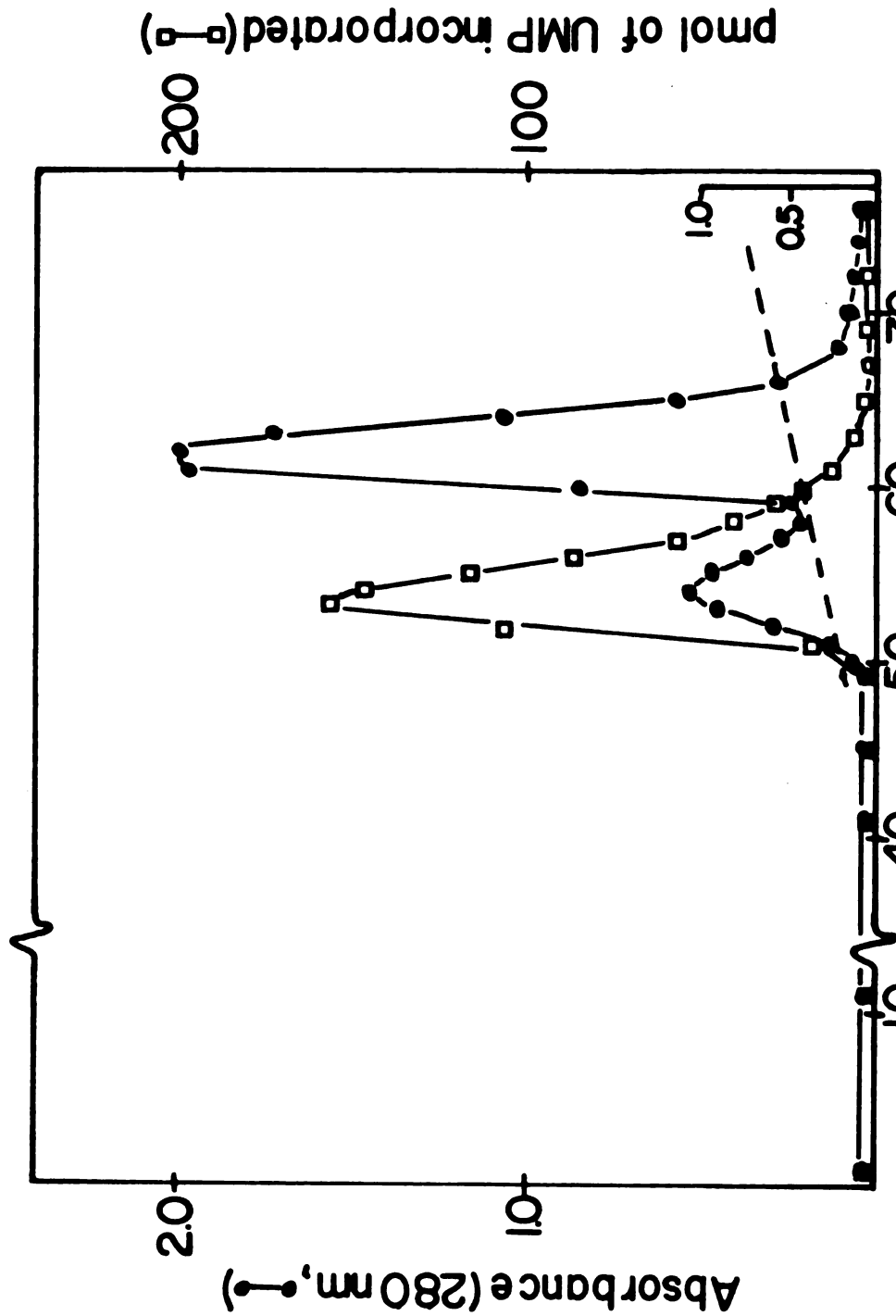

pmol of UMP incorporated (□—□)
200
100
1.0
0.5
70
60
50
40
10
Fraction number
Absorbance (280 nm, ●—●)
2.0
1.0

Figure 5

Figure 6 - Elution profile of RNA polymerase from bacteroids of R. japonicum 110 after DEAE-cellulose chromatography. Fractions (87-117) from the DNA-cellulose column were diluted with Modified TGED, applied to a column (2.0 x 6.5 cm) of DEAE-cellulose and RNA polymerase activity was eluted as described in the text. Fractions (2 ml) were collected and assayed for absorbance at 280 nm (●—●). RNA polymerase activity (□—□) was determined by assaying 10 µl samples of the column fractions. NaCl concentrations (- - -) were determined by conductivity measurements.

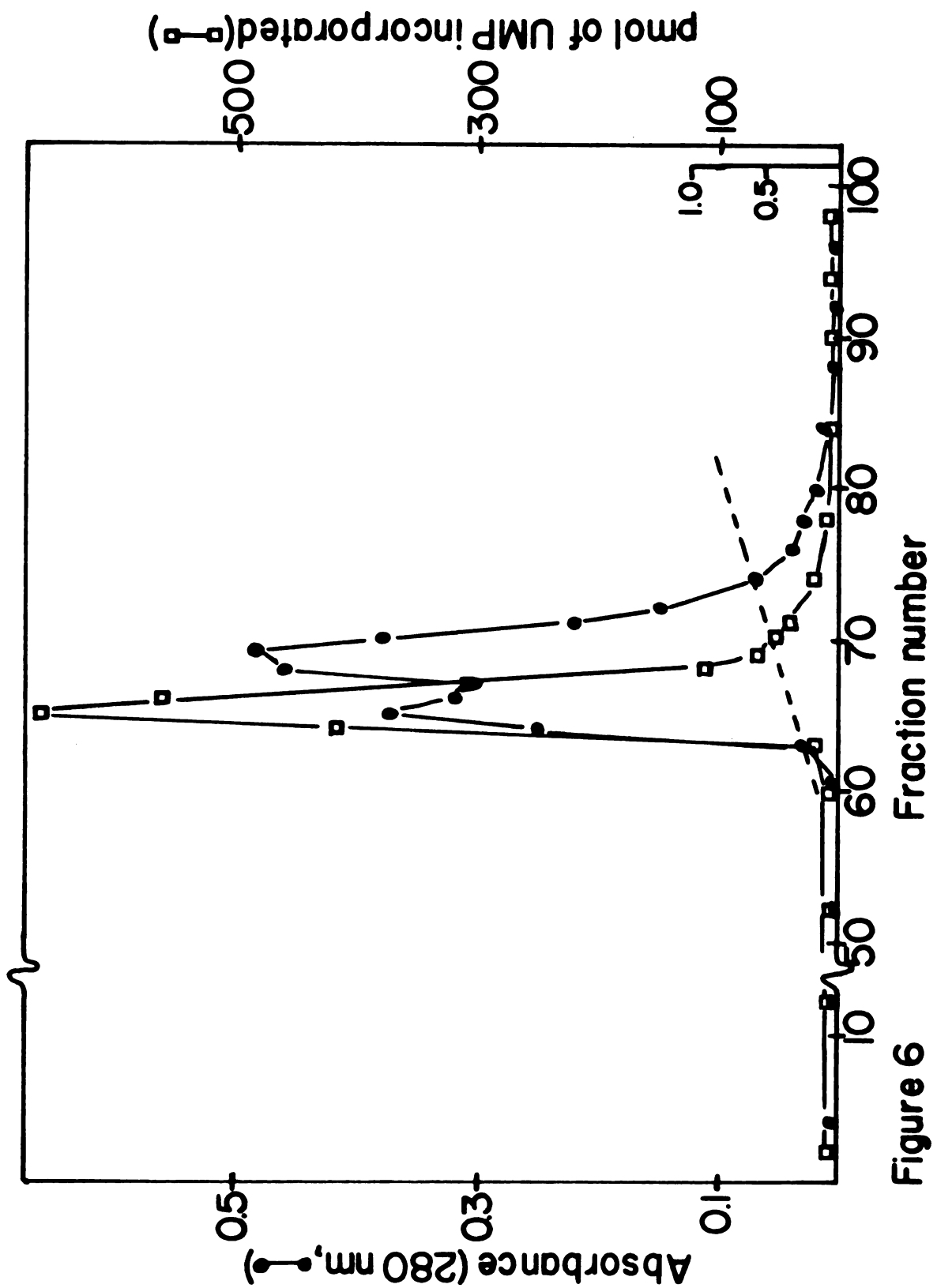

Absorbance (280 nm, ●—●)
0.5
0.3
0.1
10
50
60
70
80
90
100
Fraction number
pmol of UMP incorporated(□—□)
500
300
100
1.0
0.5

Figure 6

Table IV. Summary of Purification of RNA Polymerase From Vegetative Cells and Bacteroids

| Purification Step[a] | Total Protein[b] (mg) | Total Activity[c] (U) | Specific Activity (U/mg) |
|---|---|---|---|
| A. Vegetative Cells | | | |
| Crude supernatant | 1950 | 596 | 0.3 |
| 2 M NaCl supernatant | 459 | 268 | 0.6 |
| DNA-cellulose chromatography | 21 | 370 | 17.5 |
| DEAE-cellulose chromatography | 7 | 792 | 114.3 |
| B. Bacteroids | | | |
| Crude supernatant | 1073 | 19 | 0.02 |
| 2 M NaCl supernatant | 250 | 58 | 0.2 |
| DNA-cellulose chromatography | 6 | 347 | 54.3 |
| DEAE-cellulose chromatography | 4 | 445 | 120.3 |

(a) purification of RNA polymerase from both vegetative cells or bacteroids was performed using 20 g cells.

(b) protein was determined by the method of Lowry et al. (122). Bovine serum albumin was used as a standard.

(c) one unit of RNA polymerase activity is defined as the incorporation of one nmol of UMP into TCA precipitable material in 10 min at 37 C.

properties of RNA polymerase isolated from both sources were similar at each stage of the purification.

RNA polymerase activity from both vegetative cells and bacteroids was unstable. The addition of 25% glycerol to all chromatography buffers appeared to stabilize enzyme activity. After DEAE-cellulose chromatography, RNA polymerase was stored at -20 C. Under these conditions, 80% of the original activity was lost within 6-10 weeks.

Additional purification of RNA polymerase from vegetative cells and bacteroids of R. japonicum was attempted using preparative glycerol gradient ultracentrifugation (64), heparin-agarose chromatography (130), and agarose A 1.5 M chromatography (64). In all cases, the enzyme activity recovered from the purification was unstable and was lost rapidly. RNA polymerase from both sources also lost activity when subjected to dialysis. Dialysis of RNA polymerase in Modified TGED + 0.5 M NaCl for 24 h at 4 C resulted in the loss of 75% of the original RNA polymerase activity.

## Subunit structure of RNA polymerase

The subunit composition of RNA polymerase from vegetative cells and bacteroids of R. japonicum was evaluated after DEAE-cellulose chromatography by SDS-polyacrylamide gel electrophoresis (Figure 7). The protein banding patterns of RNA polymerase from both sources were similar (Figure 7, lanes 1,2) and resembled the banding pattern of RNA polymerase purified from E. coli (Figure 7, lane 3). Several minor constituents also appeared to co-purify with RNA polymerase from R. japonicum at each stage of the purification. The estimated molecular weights for the major protein bands from R. japonicum RNA polymerase corresponding to the $\beta\beta'$, $\sigma$ , and $\alpha$ subunits of E. coli were 170 K, 82 K and 40 K, respectively. These three

Figure 7 - SDS-polyacrylamide gel electrophoresis of RNA polymerase isolated from vegetative cells and bacteroids of R. japonicum 110. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The individual lanes contained: (1) vegetative R. japonicum RNA polymerase (2 µg), (2) bacteroid R. japonicum RNA polymerase (2 µg), (3) E. coli RNA polymerase (2 µg). Molecular weight standards used were: phosphorylase a (92,000), catalase (57,000), aldolase (40,000), carbonic anhydrase (29,000), and RNase A (13,700).

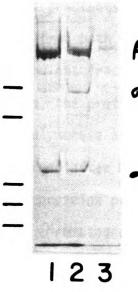



Figure 7

protein bands accounted for 85-90% of the total protein present on the SDS-polyacrylamide gel as determined by densitometry tracings of the polyacrylamide gel.

The mobility of RNA polymerase isolated from vegetative cells and bacteroids was compared by glycerol density gradient ultracentrifugation. RNA polymerase from both sources exhibited similar rates of sedimentation on 10-30% linear glycerol gradients (Figure 8). The estimated molecular weight of RNA polymerase from both vegetative cells and bacteroids was 350 K. When glycerol gradient fractions were analyzed by SDS-polyacrylamide gel electrophoresis, the protein banding pattern did not change across the peak of RNA polymerase activity (data not shown) and was the same as the pattern observed after DEAE-cellulose chromatography (Figure 7, lanes 1,2). Many of the proteins present in preparations of RNA polymerase after DEAE-cellulose chromatography (Figure 7) remained associated with the enzyme from both sources after density gradient centrifugation.

Isolation of a sigma-like protein from purified RNA polymerase

Further identification of the subunit composition of RNA polymerase was established by the method of Lowe et al. (131) using Bio-Rex 70 and DEAE-cellulose chromatography in tandem. It has been shown that core RNA polymerase ($\beta\beta'\alpha_2$) isolated from E. coli binds to Bio-Rex 70 under conditions of low ionic strength. Under these conditions, sigma does not bind to the Bio-Rex 70 matrix but does bind to the DEAE-cellulose column which follows in tandem. These procedures were used to identify a sigma-like protein from R. japonicum RNA polymerase.

Purified RNA polymerase (0.74 - 0.95 mg) was diluted with Modified TGED until the final NaCl concentration was equal to 0.1 M and was applied

Figure 8 - Glycerol gradient ultracentrifugation of RNA polymerase isolated from R. japonicum 110 vegetative cells and bacteroids. RNA polymerase from (A) vegetative cells (46 µg) and (B) bacteroids (70 µg) was applied to a 10-30% linear glycerol gradient as described in Materials and Methods. Gradient fractions (140 µl) were assayed for RNA polymerase activity. Activities of β-galactosidase and catalase were assayed according to the procedures described in Materials and Methods.

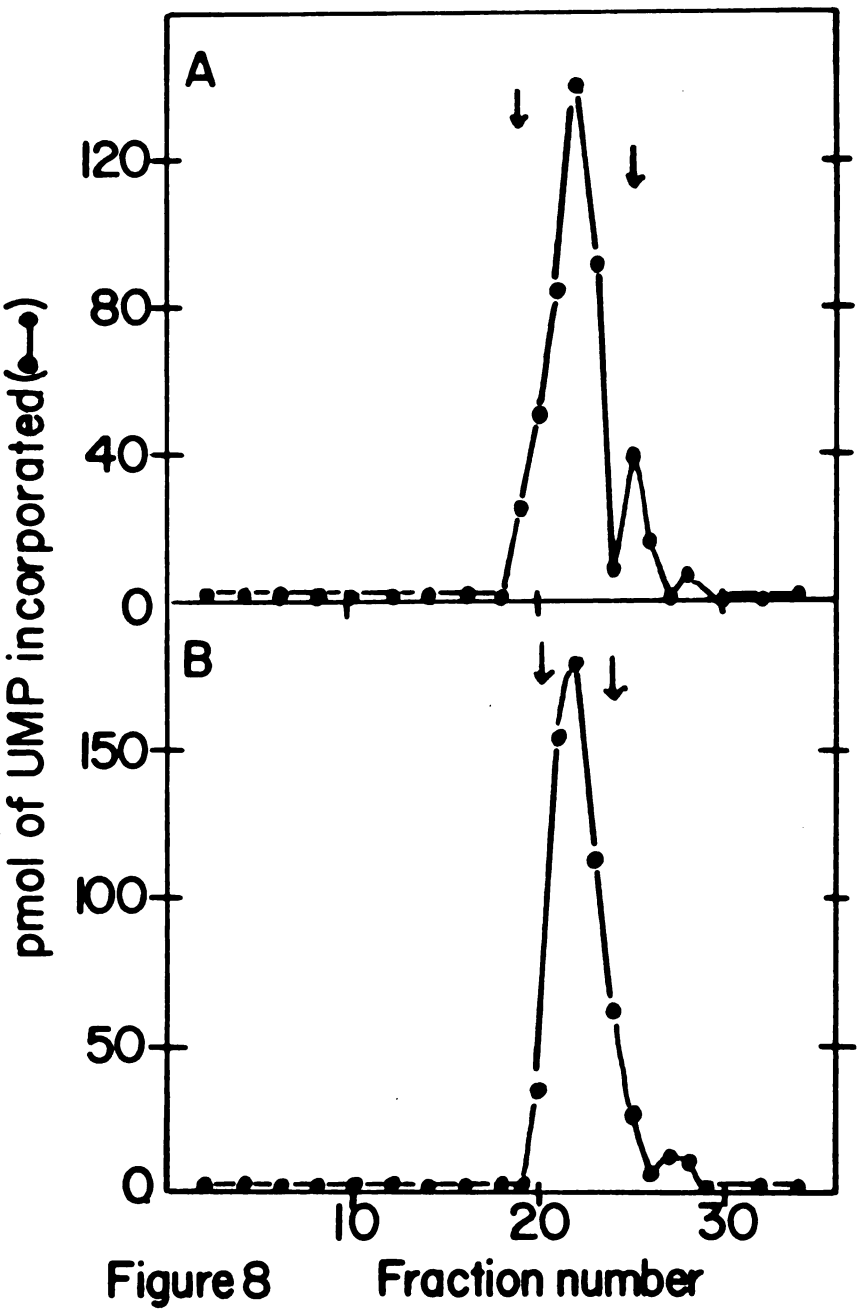

A
B
120
80
40
0
150
100
50
0
10
20
30
pmol of UMP incorporated(●—●)
Fraction number

Figure 8

Figure 9 - Bio-Rex 70 profile of RNA polymerase from vegetative cells of *R. japonicum* 110. RNA polymerase (0.74 mg) was diluted with Modified TGED, applied to a tandem column of Bio-Rex 70 (0.5 x 5.0 cm)-DEAE-cellulose (0.3 x 3.0 cm) and RNA polymerase was eluted as described in the text. Fractions (1 ml) were collected and monitored for absorbance at 280 nm (●—●). RNA polymerase activity (□—□) was determined by assaying 10 µl samples of the column fractions.

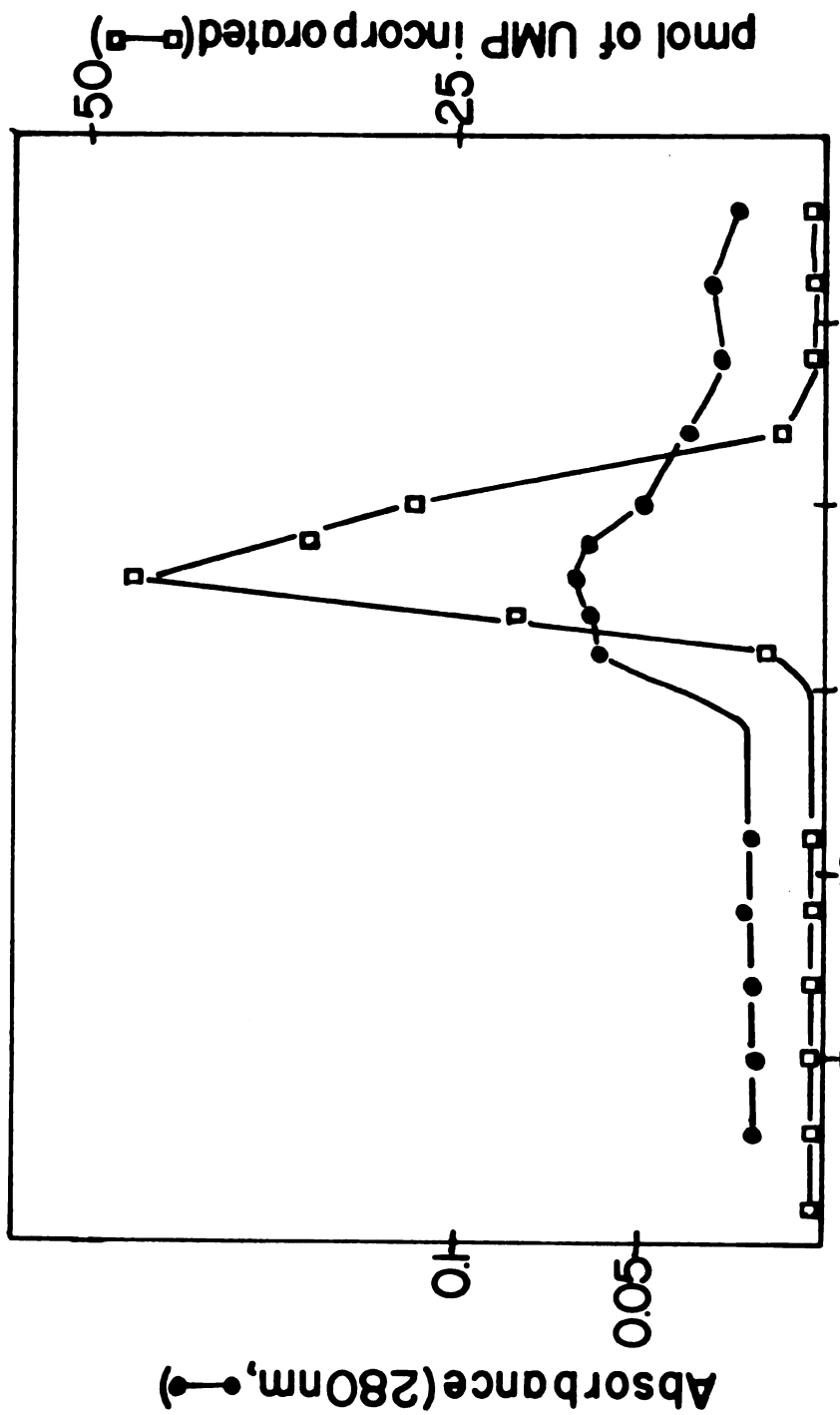

pmol of UMP incorporated(□—□)
50
25
Absorbance(280nm,●—●)
1.0
0.05
5
10
15
20
25
Fraction number

Figure 9

Figure 10 - Bio-Rex 70 profile of RNA polymerase from bacteroids of R. japonicum 110. RNA polymerase (0.95 mg) was diluted with Modified TGED, applied to a tandem column of Bio-Rex 70 (0.5 x 5.0 cm)-DEAE-cellulose (0.3 x 3.0 cm) and RNA polymerase was eluted as described in the text. Fractions (1 ml) were collected and monitored for absorbance at 280 nm (●—●). RNA polymerase activity (□—□) was determined by assaying 10 µl of the column fractions.

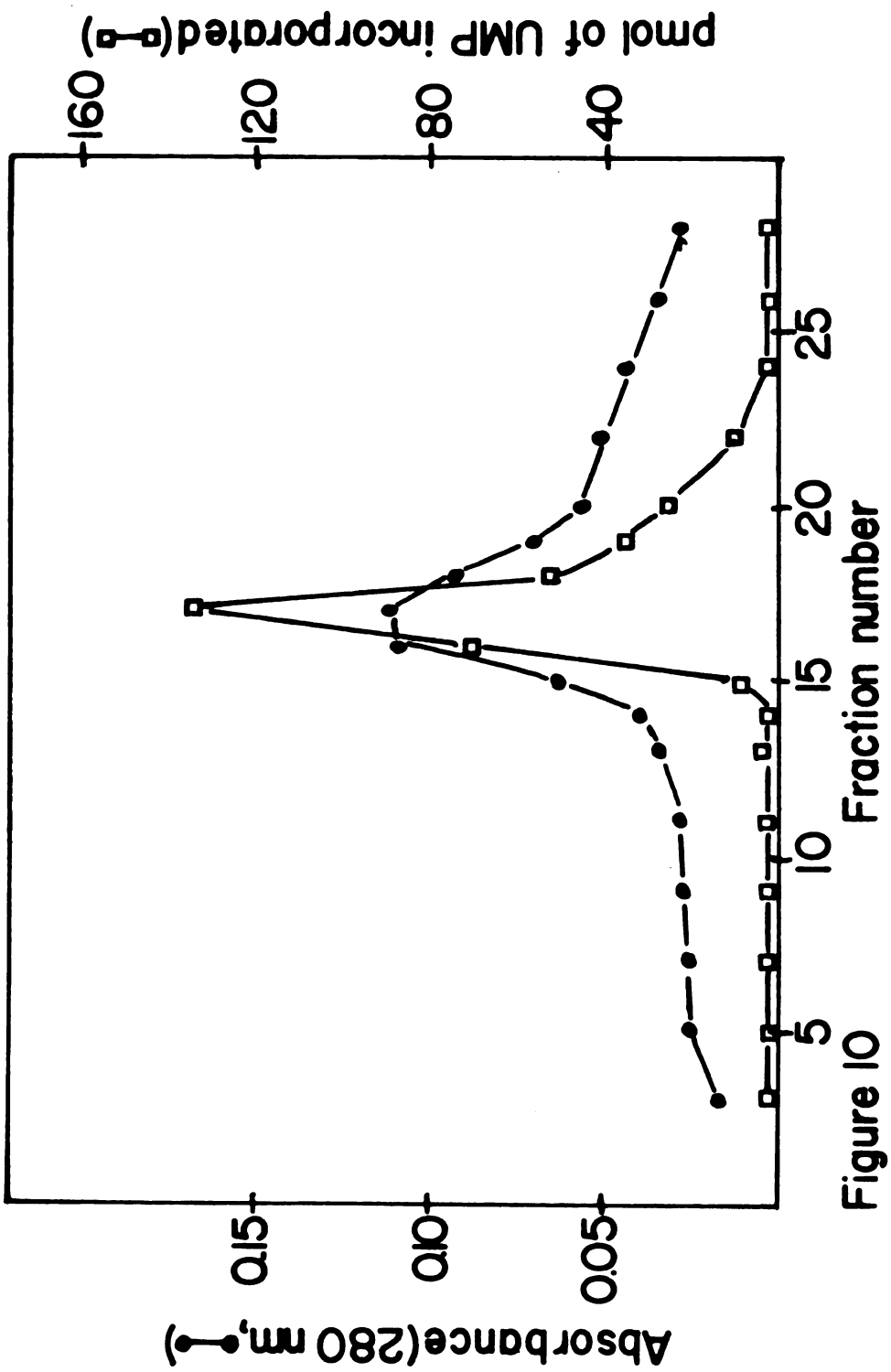

pmol of UMP incorporated(□—□)
160
120
80
40
Absorbance(280 nm,●—●)
0.15
0.10
0.05
5
10
15
20
25
Fraction number

Figure 10

to a Bio-Rex 70 (0.5 cm x 5 cm)-DEAE-cellulose (0.3 cm x 3 cm) tandem column. The tandem column was washed with 5 ml of Modified TGED + 0.1 M NaCl and the two columns were separated. Protein was eluted from the DEAE-cellulose column with 10 ml of Modified TGED + 0.5 M NaCl. Fractions were monitored by measuring absorbance at 280 nm. Protein was eluted from the Bio-Rex 70 column with a 30-ml linear gradient from 0.1 M to 0.5 M NaCl in Modified TGED. Fractions were monitored by measuring absorbance at 280 nm and by assaying for RNA polymerase activity on calf thymus DNA (Figures 9, 10) using the standard RNA polymerase assay.

Based on analysis by SDS-polyacrylamide gel electrophoresis (Figure 11, lane 1), one major protein was eluted from the DEAE-cellulose column corresponding to a molecular weight of 82 K when bacteroid RNA polymerase was applied to this tandem column. The protein banding pattern of RNA polymerase prior to and after Bio-Rex 70 column chromatography was also compared using SDS-polyacrylamide gel electrophoresis, and the major difference observed was the absence of the 82 K protein after the Bio-Rex step (Figure 11, lane 2). Similar results were obtained using RNA polymerase isolated from vegetative cells (Figure 11, lanes 3, 4). On this basis, the 82 K protein was tentatively classified as a sigma-like protein for R. japonicum RNA polymerase.

Figure 11 - SDS-polyacrylamide gel electrophoresis of protein eluted from Bio-Rex 70 and DEAE-cellulose columns for RNA polymerase from vegetative cells and bacteroids of R. japonicum 110. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The individual lanes contained: (1,2) protein eluted from the DEAE-cellulose and Bio-Rex 70 column, respectively, for RNA polymerase from bacteroids, (3,4) protein eluted from the DEAE-cellulose and Bio-Rex 70 column, respectively, for RNA polymerase from vegetative bacteria. Molecular weight standards used were: phosphorylase a (92,000), catalase (57,000), aldolase (40,000), carbonic anhydrase (29,000), and RNase A (13,700).

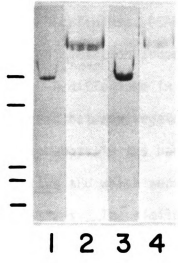



Figure II

# DISCUSSION

RNA polymerase was purified from vegetative cells and bacteroids of R. japonicum 110. The physical properties of the holoenzyme from both cell types appeared similar according to the following criteria: 1) the final specific activities after DEAE-cellulose chromatography, 2) the elution profiles after DNA-cellulose, DEAE-cellulose and Bio-Rex 70 chromatography, 3) the sedimentation properties using glycerol density gradient centrifugation. A difference in the initial specific activity of RNA polymerase was observed between vegetative cells and bacteroids. This may have been due to components in the bacteroid extract which interferred with RNA synthesis in vitro and which were separated from the enzyme during the latter purification steps. The similarity of the final specific activities of RNA polymerase from both cell types after DEAE-cellulose chromatography is consistent with this interpretation.

The purified enzyme from both sources was unstable, remaining active for only 6-10 weeks at -20 C, in contrast to E. coli RNA polymerase which can be stored stably for much longer periods of time (65). RNA polymerase activity from both sources was also inactivated by dialysis, preventing concentration of the enzyme in this manner.

The subunit composition of the enzyme from both vegetative cells and bacteroids was similar after DEAE-cellulose and Bio-Rex 70 chromatography, as determined by SDS-polyacrylamide gel electrophoresis. RNA polymerase from both forms appeared to have the $\beta\beta'\alpha_2\sigma$ structure typical of many procaryotic RNA polymerases (62) and the mobility of the major subunits corresponded to those of E. coli RNA polymerase. A few minor constituents appeared to co-purify with the enzyme from both vegetative cells and bac-

teroids at each stage of the purification. These proteins are either minor contaminants or associated polypeptides which have an, as yet, undefined role *in vivo*. RNA polymerase has also been purified from vegetative cells of *R. leguminosarum* (132) and the molecular weights of the subunits are similar to those of *R. japonicum*.

The 82 K protein was identified as a sigma-like factor for RNA polymerase from both vegetative cells and bacteroids of *R. japonicum*. This was based on both its elution properties using Bio-Rex 70 and DEAE-cellulose chromatography and the transcriptional properties of the holoenzyme vs. core RNA polymerase which will be discussed in Chapter II. The sigma subunit of the enzyme has been identified for RNA polymerase from a number of procaryotic species (133). This peptide appears to fall into one of two molecular weight classes, either 90 K or 55 K, when comparing the enzyme from different bacterial species. The 82 K sigma-like protein of *R. japonicum* RNA polymerase appears to fall into the 90 K class.

The 82 K protein was present in RNA polymerase preparations from both vegetative cells and bacteroids. While bacteroids isolated from nodules were contaminated with some vegetative bacteria, greater than 80% of the bacteria in 40 to 50-day-old soybean nodules have differentiated into bacteroids (134). Therefore, it is unlikely that the presence of the 82 K protein in bacteroid preparations is due to contamination from vegetative bacteria.

In this chapter, a purification for RNA polymerase from both vegetative cells and bacteroids of *R. japonicum* 110 has been presented. The physical properties of the enzyme from both sources appears similar based on the chromatographic properties which were analyzed. The subunit composition was also similar for RNA polymerase from vegetative cells and

bacteroids, and shared features common to other procaryotic polymerases. However, a comparison of the physical characteristics of RNA polymerase from these two sources is only significant when made in conjunction with analysis of the transcriptional specificity of the enzyme. Therefore, a characterization of the transcriptional properties of RNA polymerase from both forms of R. japonicum 110 is presented in Chapter II.

# CHAPTER II

## TRANSCRIPTIONAL PROPERTIES OF RNA POLYMERASE FROM VEGETATIVE CELLS AND BACTEROIDS OF *RHIZOBIUM JAPONICUM*

The transcriptional control mechanism(s) which govern the expression of *nif* and symbiotic genes during bacteroid development is not well understood. The transcriptional properties of purified RNA polymerase from both sources of *R. japonicum* 110 were examined using a variety of exogenous templates. The kinetic parameters of RNA synthesis were examined for RNA polymerase from both vegetative cells and bacteroids and were found to be similar. RNA polymerase from both sources was also found to utilize the early promoters on T7 DNA, *in vitro*. The transcription of cloned *nif* genes *in vitro* was also examined. RNA polymerase from both vegetative cells and bacteroids of *R. japonicum* was able to recognize *nif*-specific promoters on pRJ676 indicating that no positive regulatory factor is needed for the transcription of these genes *in vitro*.

# RESULTS

## Characterization of the RNA polymerase reaction

RNA polymerase purified from vegetative cells and bacteroids catalyzed RNA synthesis *in vitro*. This reaction was dependent on a DNA template, the presence of nucleoside triphosphates and $Mg^{+2}$ (Table V). RNA synthesis increased in a linear manner with enzyme concentration up to 0.3 mg/ml of protein. No labeled TCA-precipitable product was recovered if RNase A or DNase I was included in the reaction mixture.

RNA synthesis was also sensitive to heparin and rifampicin, both known inhibitors of initiation of RNA synthesis. At a heparin concentration of 100 µg/ml, RNA polymerase activity from either form of *R. japonicum* was inhibited 98% over that of the control. RNA synthesis was also sensitive to low concentrations of rifampicin (Figure 12). Some preparations of RNA polymerase isolated from bacteroids were more resistant to low levels of rifampicin ($< 0.5$ µg/ml) than RNA polymerase isolated from vegetative cells. However, this was not a consistent obsevation with many of the bacteroid RNA polymerase preparations.

## RNA synthesis on T7 DNA

The specificity of promoter recognition by *R. japonicum* RNA polymerase was characterized using wild type T7 DNA as a template. Wiggs *et al.* (125) have shown that, *in vitro*, RNA polymerase from several bacterial orders recognize the same subset of early promoters on T7 DNA utilized by *E. coli* RNA polymerase *in vivo*. To determine if these promoters were also recognized by RNA polymerase from both vegetative cells and bacteroids of *R. japonicum*, the $^{32}P$-labeled RNA synthesized *in vitro* was analyzed by

Table V. Requirements of the RNA Polymerase Reaction

| Reaction Mixture | % Activity | |
|---|---|---|
| | Vegetative Bacteria | Bacteroids |
| Complete | 100 | 100 |
| -ATP | 5 | 4 |
| -CTP | 0 | 7 |
| -GTP | 2 | 4 |
| -DNA | 0 | 0 |
| -$MgCl_2$ | 3 | 0 |
| + 1 μg RNase A | 3 | 3 |
| + 1 μg DNase I | 0 | 2 |
| + 1 μg heparin | 2 | 0 |

RNA polymerase reactions were carried out as described in Materials and Methods for the standard RNA polymerase assay, where 3 μg of purified RNA polymerase was added to each reaction mixture. RNase A, DNase I and heparin were added to the reaction mixture before beginning the incubation at 37 C. In the complete reaction mixture, 100% activity represents 122 and 174 pmol of UMP incorporated in 10 min at 37 C for bacterial and bacteroid RNA polymerase, respectively. The above values represent the average of 4 separate experiments which were performed in duplicate.

Figure 12 - Rifampicin inhibition of RNa polymerase from vegetative cells and bacteroids of R. japonicum. RNA polymerase assays were carried out as described in Materials and Methods. Rifampicin was added to the reaction mixture before the beginning of the incubation at 37 C. In a complete reaction mixture, 100% activity represents 68 and 76 pmol of UMP incorporated in 10 min at 37 C for bacterial and bacteroid RNA polymerase, respectively.

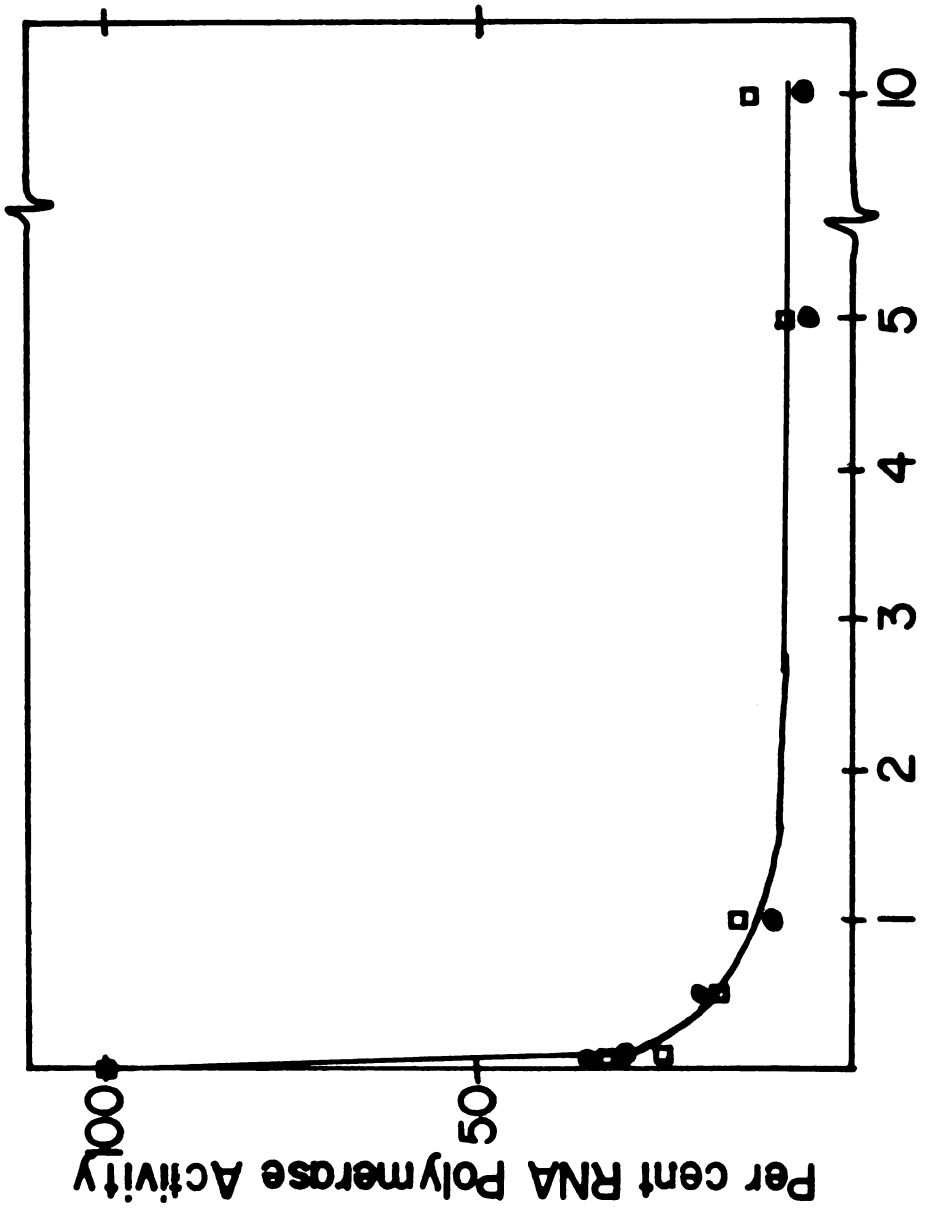

Per cent RNA Polymerase Activity
100
50
1
2
3
4
5
10
[Rifampicin], ug/ml

Figure 12

agarose-acrylamide gel electrophoresis. A single transcript with an apparent molecular weight of $2.5 \times 10^6$ was synthesized *in vitro* by RNA polymerase from both sources (Figure 13).

The RNAs synthesized using T7 D111 DNA were also characterized. D111 is a mutant of T7 in which two of the three A promoters have been deleted. Use of this DNA as a template, *in vitro*, allows one to analyze for the use of other "early" promoters on T7 DNA by RNA polymerase. When T7 DNA was used as a template *in vitro* for RNA polymerase from *R. japonicum*, three transcripts with apparent molecular weights of $2.5 \times 10^6$, $1.7 \times 10^6$, and $7.5 \times 10^4$ were synthesized (Figure 14). These three transcripts presumably correspond to the A, C and D transcripts synthesized by *E. coli* RNA polymerase on T7 DNA. The D transcript is not easily seen in Figure 14, but its presence was verified by analyzing an autoradiography after a longer exposure. There was no difference in the transcription pattern between *E. coli* RNA polymerase and the *R. japonicum* enzyme on either wild type or D111 T7 DNA.

Using the method of Chamberlin *et al.* (11) several kinetic parameters of bacterial RNA polymerases can be estimated including the elongation rate, efficiency of transcriptional termination, as well as the amount of active enzyme which is present in a given RNA polymerase preparation. A requirement for this type of analysis is that RNA polymerase must initiate almost exclusively at the A promoter when wild type T7 DNA is used as a template *in vitro*. This was shown to be the case for purified RNA polymerase from vegetative cells and bacteroids of *R. japonicum* (Figure 13). Therefore, a time course of RNA synthesis on T7 DNA can be used to calculate the above kinetic parameters for *R. japonicum* RNA polymerase.

The elongation rate can be calculated from the amount of time it

Figure 13 - Electorphoretic analysis of RNAs synthesized on wild type T7 DNA using RNA polymerase from E. coli and from vegetative cells and bacteroids of R. japonicum. RNA polymerase reactions were carried out using pre-binding conditions on wild type T7 DNA as described in Materials and Methods. RNA samples containing approximately 50,000 cpm were analyzed by agarose-acrylamide gel electrophoresis using 0.6% agarose-2% acrylamide gels. Lane (a) E. coli RNA polymerase, (b) R. japonicum vegetative RNA polymerase, (c) R. japonicum bacteroid RNA polymerase.

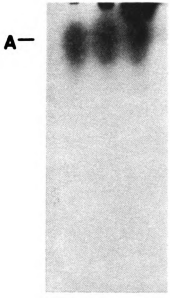



Figure 13

Figure 14 - Electrophoretic analysis of RNAs synthesized on D111 T7 DNA using RNA polymerase from E. coli and from vegetative cells and bacteroids of R. japonicum. RNA polymerase reactions were carried out using pre-binding conditions on D111 T7 DNA as described in Materials and Methods. RNA samples containing approximately 50,000 cpm were analyzed by agarose-acrylamide gel electrophoresis using 0.6% agarose-2% acrylamide gels. Lane (a) E. coli RNA polymerase, (b) R. japonicum vegetative RNA polymerase, (c) R. japonicum bacteroid RNA polymerase.

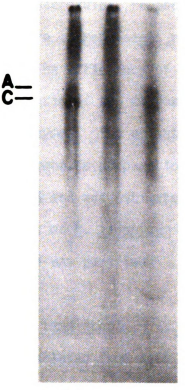



Figure 14

takes to complete the first linear phase of RNA synthesis (Figure 15) and was estimated to be 12 and 13 base pairs per second for RNA polymerase from vegetative cells and bacteroids, respectively. The second linear phase of RNA synthesis in Figure 15 represents read-through of a transcriptional termination signal. The efficiency of transcriptional termination can be determined from the ratio of the slopes of phase 2/phase 1 and was found to be 85-88% for both enzymes. The amount of active enzyme in the vegetative and bacteroid RNA polymerase preparations can be calculated using the data presented in Figure 15 and was estimated to be 13% and 27%, respectively. The kinetic parameters of R. japonicum RNA polymerase were determined three weeks after the enzyme was purified.

Activity on exogenous templates

RNA polymerase isolated from both sources of R. japonicum was tested for its ability to direct RNA synthesis in vitro, using a variety of exogenous templates (Table VI). Bacteroid RNA polymerase preparations synthesized twice as much RNA using P22, lambda, and T7 DNA as templates as the enzyme from vegetative cells. RNA polymerase from both sources utilized calf thymus DNA and poly d(AT) to similar extents.

RNA polymerase activity on T4 DNA

The role of the 82 K protein as a sigma-like factor for R. japonicum RNA polymerase was tested using T4 DNA as a template, in vitro. Core RNA polymerase ($\beta\beta'\alpha_2$) isolated from E. coli has a reduced ability to synthesize RNA using T4 DNA as a template, when compared to the holoenzyme (9, 131). RNA polymerase isolated from R. japonicum vegetative cells and bacteroids was eluted from a Bio-Rex 70 column in a manner similar to E.

Table VI. Activity of RNA Polymerase from Vegetative Cells and Bacteroids of *R. japonicum* on Exogenous Templates

| Template | RNA Polymerase Activity | |
|---|---|---|
| | Vegetative Bacteria | Bacteroids |
| | (pmol of UMP incorporated) | |
| Native calf thymus DNA | 88 | 84 |
| Denatured calf thymus DNA | 28 | 30 |
| P22 DNA | 18 | 32 |
| Lambda DNA | 27 | 56 |
| T7 DNA | 41 | 67 |
| Poly d(AT) | 225 | 175 |

RNA polymerase reactions were carried out in a manner similar to those described in the Materials and Methods for the standard RNA polymerase assay using 2 µg of purified vegetative or bacteroid RNA polymerase per assay. The following amounts of purified DNA's were used: native calf thymus DNA (7.5 µg), denatured calf thymus DNA (7.5 µg), P22 DNA (5.5 µg), lambda DNA (1.6 µg), T7 DNA (1.25 µg), poly d(AT) (7.5 µg). The above values represent the average of 2 separate experiments which were performed in duplicate.

Figure 15 - Kinetics of RNA synthesis on T7 DNA using RNA polymerase from vegetative cells and bacteroids of R. japonicum. RNA polymerase from (A) vegetative cells (36 µg) and (B) bacteroids (32 µg) of R. japonicum was incubated with wild type T7 DNA at 30 C. RNA synthesis was carried out as described (119). Aliquots of 100 µl were taken at the indicated time points and analyzed for [$^{14}$C]AMP incorporation into TCA-precipitable material.

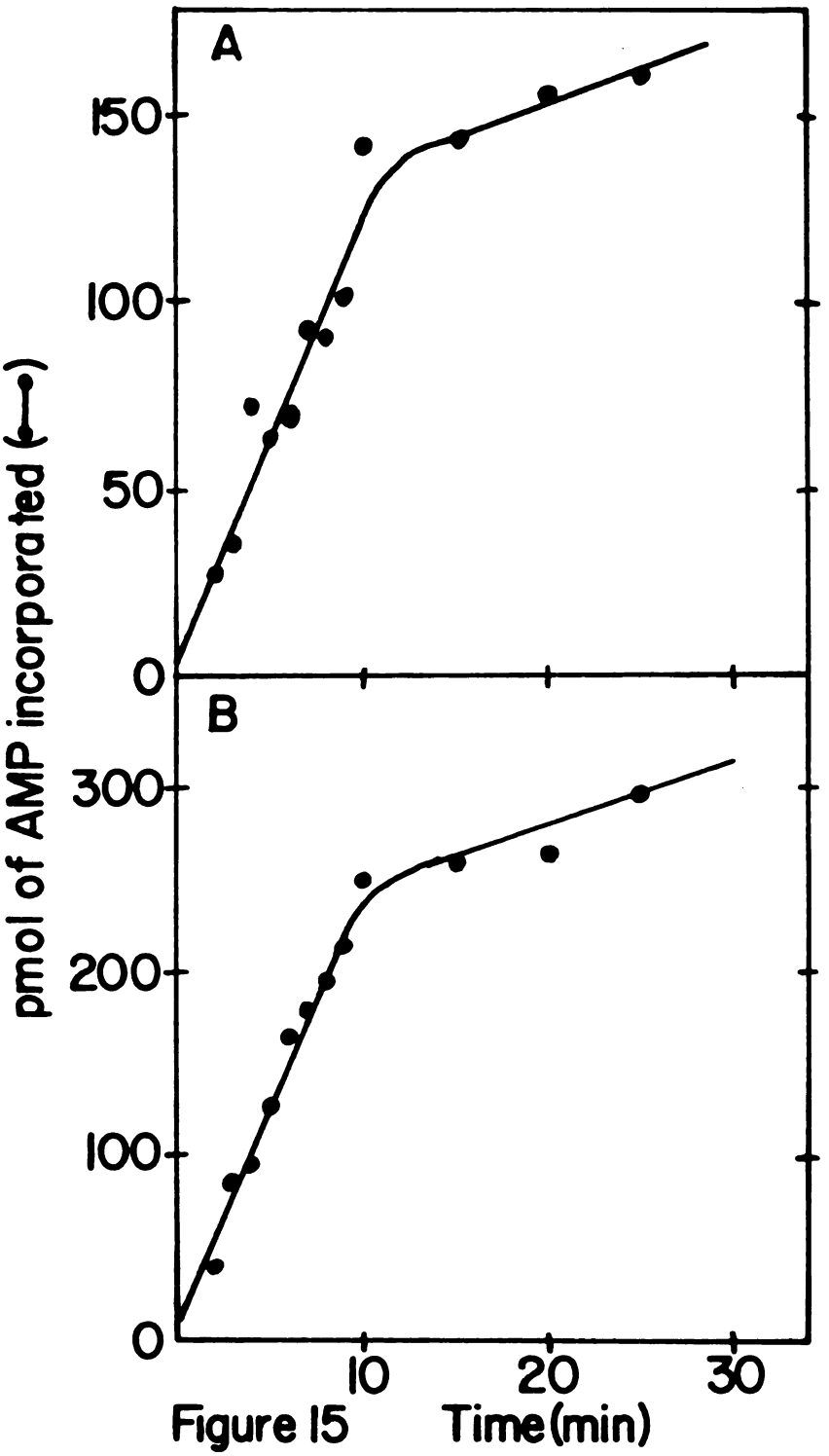

A
B
150
100
50
0
300
200
100
0
10
20
30
pmol of AMP incorporated (•—•)
Time(min)

Figure 15

coli core RNA polymerase and was tested for its ability to direct RNA synthesis on T4 DNA. The activity of the enzyme after Bio-Rex 70 chromatography was decreased when compared to the corresponding holoenzyme for RNA polymerase from both forms of R. japonicum (Table VII).

Reconsititution experiments with purified sigma factor were difficult due to the apparent instability of the protein. When purified E. coli sigma was added to core RNA polymerase from either E. coli or R. japonicum, a 2-3 fold stimulation of RNA synthesis was seen. However, this activity is much lower than that observed in reconstitution experiments by other workers (131). An increase in RNA polymerase activity was also seen in most experiments using the 82 K protein from vegetative cells or bacteroids with core enzymes from either form of R. japonicum. This stimulation was not always seen with E. coli core RNA polymerase and the 82 K protein from either form of R. japonicum.

Recognition of nif-specific promoters

The ability of RNA polymerase from vegetative cells and bacteroids of R. japonicum to recognize nif-specific promoters was characterized using recombinant plasmids containing the nif KD operon of R. japonicum (113) and the nif KDH operon of R. meliloti (57) and K. pneumoniae (114). In a southern hybridization experiment, $^{32}$P-labeled RNA was synthesized in vitro using pRJ676 (113), a plasmid containing the R. japonicum nif genes, as a DNA template. This RNA was purified by ethanol precipitation and hybridized to electrophoretically separated fragments of restricted pRJ676 (Figure 16). RNA synthesized in vitro using E. coli RNA polymerase or RNA polymerase from either form of R. japonicum was found to hybridize to both the vector, pBR322, and the DNA insert of pRJ676.

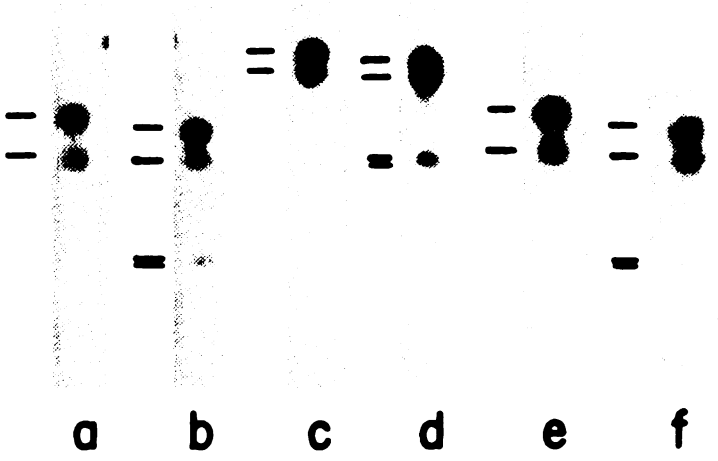
Table VII. Transcriptional Activity of *E. coli* and *R. japonicum* Holoenzyme and Core RNA Polymerase on T4 DNA

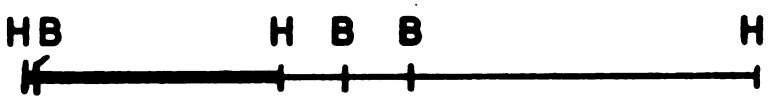
| A. Source of Enzyme | RNA Polymerase Activity | | |
|---|---|---|---|
| | holoenzyme | core enzyme | sigma |
| | (pmol of UMP incorporated) | | |
| *E. coli* | 388 | 54 | 0.4 |
| *R. japonicum* bacteria | 143 | 43 | 0.7 |
| *R. japonicum* bacteroids | 144 | 72 | 0 |

| B. Source of core RNA polymerase | RNA Polymerase Activity | | |
|---|---|---|---|
| | *E. coli* sigma | *R. japonicum* bacterial 82 K protein | *R. japonicum* bacteroid 82 K protein |
| *E. coli* | 137 | 71 | 36 |
| *R. japonicum* bacteria | 122 | 59 | 45 |
| *R. japonicum* bacteroids | 138 | 90 | 94 |

The activity of RNA polymerase holoenzyme and core enzyme on T4 DNA was determined as described in the Materials and Methods. The amount of enzyme used for each of the assays was: *E. coli* holoenzyme - 3 µg, *E. coli* core - 1.8 µg, *E. coli* sigma - 0.7 µg, vegetative *R. japonicum* holoenzyme - 1.5 µg, vegetative *R. japonicum* core - 1.6 µg, vegetative *R. japonicum* sigma-like 82 K protein - 0.6 µg, bacteroid *R. japonicum* holoenzyme - 1.8 µg, bacteroid *R. japonicum* core - 1.5 µg, bacteroid *R. japonicum* sigma-like 82 K protein - 0.6 µg. The values presented above represent the average of the mean of two experiments.

Figure 16 - Southern hybridization analysis of RNA synthesized on pRJ676 DNA using RNA polymerase from E. coli and from vegetative cells and bacteroids of R. japonicum. RNA polymerase reactions were carried out as described in Materials and Methods. The RNA was ethanol precipitated and hybridized to restriction fragments of pRJ676 which had been immobilized on nitrocellulose filters. The lines adjacent to each lane represent the position of the DNA restriction fragments. Lanes a, c, and e contain the Hind III restriction fragments of pRJ676 and lanes b, d and f contain the restriction fragments of pRJ676 which were generated by cleavage with Hind III and Bam HI. Restriction fragments were separated by electrophoresis on 1% agarose gels. Lanes (a,b) E. coli RNA polymerase, (c,d) R. japonicum vegetative RNA polymerase, (e,f) R. japonicum bacteroid RNA polymerase.




pRJ676

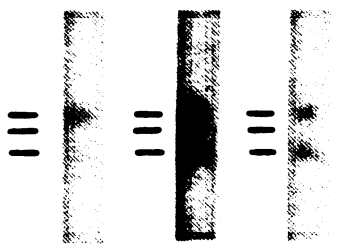
Figure 16

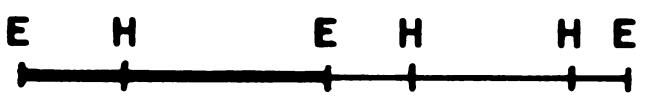
In a similar experiment, $^{32}$P-labeled RNA was synthesized *in vitro* using pRmR2, a plasmid containing the *R. meliloti nif* KDH genes (57), as a template. The three largest fragments of this plasmid, created by restriction with Eco R1 and Hind III had previously been gel purified (115). These fragments were separated according to size using agarose gel electrophoresis and were immobilized on nitrocellulose. When the $^{32}$P-labeled RNA synthesized from pRmR2 *in vitro* was hybridized to these filters, it was found to hybridize to two restriction fragments containing vector sequences, but not to the fragment originating from the *R. meliloti* insert (Figure 17). This fragment has been shown to contain the transcription initiation site for the *R. meliloti* KDH operon *in vivo* (135).

The plasmid pSA30 (114) containing the *K. pneumoniae* KDH operon was also used as a DNA template *in vitro* to direct RNA synthesis. In this experiment, the plasmid was restricted with Eco R1 before it was used as a DNA template *in vitro*. The $^{32}$P-labeled RNA was again ethanol precipitated and hybridized to electrophoretically separated fragments of pSA30 (Figure 18). The RNA synthesized *in vitro* was found to hybridize to both the vector and the *Klebsiella* insert DNA. There was no difference in the transcription pattern when RNA polymerase from *E. coli* or either form of *R. japonicum* was used.

From the southern hybridization experiment using *R. japonicum nif* DNA, it was not possible to determine with certainty whether RNA synthesis had initiated at promoters present on the *nif* DNA insert. To analyze this question more carefully, the promoter specificity of RNA polymerase from *E. coli* and both forms of *R. japonicum* was characterized using ternary transcription complex analysis (117). With this approach, restriction fragments containing promoters can be more clearly identified by electro-

Figure 17 - Southern hybridization analysis of RNA synthesized on pRmR2 DNA using RNA polymerase from E. coli and from vegetative cells and bacteroids of R. japonicum. RNA polymerase assays were carried out as described in Materials and Methods. The RNA was ethanol precipitated and hybridized to restriction fragments of pRmR2 which had been immobilized on nitrocellulose filters. The lines adjacent to each lane represent the position of the DNA restriction fragments. Lanes a, b and c contain the three largest restriction fragments of pRmR2 generated by cleavage with Hind III and Eco RI. Restriction fragments were separated by electrophoresis on 1% agarose gels. Lanes (a) E. coli RNA polymerase, (b) R. japonicum vegetative RNA polymerase, (c) R. japonicum bacteroid RNA polymerase.

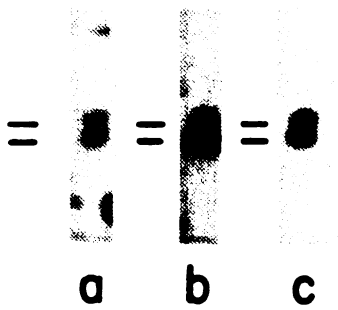


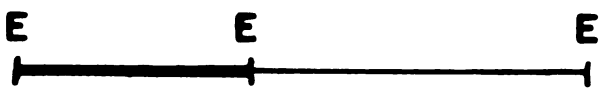




pRmR2

Figure 17

Figure 18 - Southern hybridization analysis of RNA synthesized on pSA30 DNA using RNA polymerase from E. coli and from vegetative cells and bacteroids of R. japonicum. RNA polymerase assays were carried out on pSA30 DNA fragments generated by cleavage with Eco RI as de- scribed in Materials and Methods. The RNA was ethanol precipitated and hybridized to Eco RI restriction fragments of pSA30 DNA which had been immobilized on nitrocellulose filters. The lines adjacent to each lane represent the position of the DNA restriction fragments. Restriction fragments were separated by electrophoresis on 1% agarose gels. Lanes (a) E. coli RNA polymerase, (b) R. japonicum vegetative RNA polymerase, (c) R. japonicum bacteroid RNA polymerase.



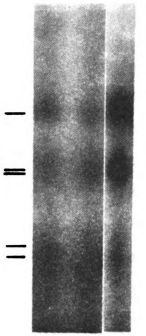

pSA30

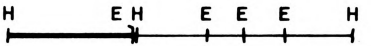
Figure 18

phoretic separation of the DNA-RNA polymerase-nascent RNA ternary complexes in agarose gels.

RNA polymerase from E. coli and both forms of R. japonicum was found to initiate RNA synthesis on both vector and insert DNA restriction fragments of pRJ676 (Figure 19). RNA polymerase from both sources of R. japonicum utilized promoters on pBR322 and on a 2.7 kb and a 1.2 kb restriction fragment of the insert of pRJ676. The transcription pattern for E. coli RNA polymerase was similar to that of RNA polymerase from R. japonicum.

Figure 19 - Electrophoretic analysis of ternary transcription complexes formed using E. coli and R. japonicum RNA polymerase and pRJ676 DNA . RNA polymerase reactions were carried out using the pre-cut protocol as described in Materials and Methods. RNA samples containing 10,000-40,000 cpm were analyzed by electrophoresis on 1.2% agarose gels. Lane (a) R. japonicum vegetative RNA polymerase (1 µg), (b) R. japonicum bacteroid RNA polymerase (1 µg), (c) E. coli RNA polymerase (1 µg).




pRJ676

Figure 19

# DISCUSSION

The transcriptional properties of RNA polymerase from vegetative cells and bacteroids of R. japonicum 110 have been investigated. The requirements for RNA synthesis were similar to those of other procaryotic RNA polymerases and the enzyme from both sources was found to be sensitive to rifampicin and heparin. Several exogenous DNA templates were utilized by RNA polymerase in vitro. The enzyme isolated from bacteroids synthesized twice as much RNA as the enzyme isolated from bacteria when phage DNA was used as a template. This difference in template utilization is most likely due to twice as much active RNA polymerase in the bacteroid preparation, as measured by the method of Chamberlin et al. (11).

The function of the 82 K protein was investigated for RNA polymerase from both vegetative cells and bacteroids of R. japonicum. The 82 K protein appeared to act like a "sigma" protein for R. japonicum RNA polymerase based on the reduced ability of the enzyme from either source to transcribe T4 DNA when the 82 K protein was removed. The decrease in RNA polymerase activity for R. japonicum RNA polymerase after Bio-Rex chromatography was not as dramatic as that observed for the E. coli enzyme. This may be due to different transcriptional properties of R. japonicum core RNA polymerase on T4 DNA or a contamination of the core RNA polymerase preparations with some active sigma. Purified E. coli sigma was able to stimulate the core enzymes from E. coli and both forms of R. japonicum. While the 82 K protein stimulated the RNA polymerase activity of the core enzyme from either source of R. japonicum, it had little or no effect on E. coli core RNA polymerase.

Promoter utilization was measured directly on T7 DNA by analyzing the

$^{32}$P-labeled RNAs synthesized during an *in vitro* assay. There did not appear to be any major differences between the two sources of *R. japonicum* RNA polymerase in promoter utilization and in both cases, the enzyme used the same early promoters as *E. coli* RNA polymerase. Only one major transcript was synthesized when wild type T7 DNA was used as a template while three RNA species corresponding in size to the A, C and D transcripts were synthesized when D111 T7 DNA was the template. This pattern is consistent with that found with RNA polymerases isolated from a wide variety of bacterial orders (125) and indicates that preparations of RNA polymerase from vegetative cells and bacteroids of *R. japonicum* contain an enzyme with a promoter recognition mechanism similar to that of other procaryotic RNA polymerases.

Since RNA polymerase isolated from both forms of *R. japonicum* was found to utilize the A promoter exclusively on wild type T7 DNA, the elongation rate and efficiency of termination could be estimated, using the method of Chamberlin *et al.* (11). These kinetic parameters were similar for RNA polymerase isolated from both cell types and were within the range of those reported for other procaryotic RNA polymerases (11).

Krol *et al.* (47) have demonstrated that plasmids present in *Rhizobium* containing the nitrogenase structural genes are heavily transcribed in bacteroids and not in vegetative bacteria. As has been shown in studies with *Bacillus subtilis*, it is essential to examine the transcriptional specificity of RNA polymerase on known developmentally regulated genes (107,108). The structural genes for component I of nitrogenase have been cloned from a number of *Rhizobium* species. Plasmids containing the *nif* DK genes from *R. japonicum* and the *nif* HDK genes from *R. meliloti* were used as DNA templates *in vitro* to determine if RNA polymerase isolated from

vegetative cells and bacteroids would recognize a nif-specific promoter.

Using southern hybridization analysis, RNA polymerase from both forms of R. japonicum was found to synthesize RNA which hybridized to both the vector and insert of pRJ676, which contains R. japonicum nif DNA. When a similar experiment was performed using pRmR2 as a DNA template, the RNA synthesized was found to hybridize to the vector DNA sequences but not to the restriction fragment containing the R. meliloti nif genes. While this fragment has been shown to contain the initiation site for transcription in vivo, it is possible that DNA sequences located 5' to this start site necessary for transcription in vitro are not present on pRmR2. Alternatively, it is possible that purified RNA polymerase from R. japonicum will not recognize a nif promoter from R. meliloti in vitro without the presence of additional regulatory factors. The transcriptional specificity of RNA polymerase from E. coli was found to be the same as RNA polymerase from both sources of R. japonicum on both pRJ676 and pRmR2.

Southern hybridization analysis was also used to analyze promoter utilization on pSA30. This plasmid contains the nif KDH operon of K. pneumoniae, a free-living bacteria capable of fixing nitrogen. RNA polymerase from E. coli and both forms of R. japonicum was found to synthesize RNA in vitro using the restriction fragments of pSA30 generated by cleavage with Eco R1 as templates. This RNA was found to hybridize to both the vector and insert of pSA30 indicating that both DNA restriction fragments could serve as templates for RNA synthesis in vitro.

The specificity of promoter recognition on the R. japonicum nif genes was further tested using ternary transcription complex analysis. In this experiments, RNA polymerase from both vegetative cells and bacteroids was found to recognize promoters on pBR322 and on 2 restriction fragments of

the pRJ676 insert DNA, *in vitro*. The 2.7 kb fragment contains the amino-terminal coding sequence for the *nif* D gene (113,136) and presumably a promoter for the *R. japonicum nif* DK operon. RNA polymerase from *E. coli* was also found to recognize these promoters *in vitro*. This indicates that no positive regulatory factor is necessary for the transcription of *R. japonicum nif* genes *in vitro*. Recently, Fuhrmann and Hennecke (136) have analyzed the proteins coded for by the DNA insert of pRJ676. This was accomplished by fusing subclones of this DNA sequence with strong promoters and assaying for expression of these clones in *E. coli* minicells. By this method, they detected two regions within the insert of pRJ676 which code for *R. japonicum*-specific polypeptides. One of these starts within the 2.7 kb Eco R1-Hind III restriction fragment of the plasmid which contains the *nif* D gene sequence. The other region appears to begin within the 1.2 kb Eco R1 restriction fragment of pRJ676. The restriction fragments containing the amino-terminal end of these polypeptides correlate with the restriction fragments which appear to contain promoters, based on the ternary transcription complex analysis.

In this chapter, the transcriptional properties of RNA polymerase from vegetative cells and bacteroids of *R. japonicum* have been analyzed. The transcriptional specificity of the enzyme from both sources appears to be similar on both exogenous DNA templates and cloned *nif* genes, *in vitro*. The ability of RNA polymerase from either source of *R. japonicum* to utilize specific promoters, *in vitro*, also appears to be similar to that of *E. coli* RNA polymerase. However, this may not be the case for RNA polymerase from all species of *Rhizobium*. Purified *E. coli* RNA polymerase does not appear to recognize the *nif* KDH promoter of *R. meliloti*, *in vitro* (V. Sundaresan, personal commun.). This observation may also be true for *R. japonicum* RNA

polymerase, based on the southern hybridization analysis. The structural organization of the nitrogenase genes has been found to be different in several nitrogen fixing microorganisms (57,136,137). It is possible that this difference in structural organization may reflect a difference in the transcriptional regulation between the various species of Rhizobium. It would therefore seem desirable to utilize purified RNA polymerase from the bacterial species in question when analyzing the transcriptional regulation of nif and other symbiotic genes, in vitro.

Transcriptional control of nif gene expression has been most extensively studied in K. pneumoniae (60,61). In this system, it appears that transcriptional regulation occurs on two levels. The nif LA cistron codes for polypeptides which mediate the expression of all nif operons other than their own. The nif A and L gene products function as activator and repressor molecules, respectively, and their action is independent of the other nitrogen regulatory systems in the cell.

Expression of the nif genes in K. pneumoniae is also controlled by the gln F and gln G gene products. These proteins appear to activate transcription of the nif LA operon and ensure that expression of these genes is regulated according to the level of fixed nitrogen which is available for growth. In this manner, the nif LA operon is regulated in a coordinate fashion with other genes involved in cellular nitrogen metabolism, while expression of all other nif operons is under more stringent transcriptional controls.

A similar type of regulatory system may be involved in nif expression in some Rhizobium species. The inability of E. coli or R. japonicum RNA polymerase to utilize the nif HDK genes as a template, in vitro, indicates that a positive activator similar to the Klebsiella nif A protein may be

required for transcription. Ow and Ausubel (61) have recently demonstrated that transcription of the R. meliloti nif H gene can be activated by the E. coli gln G protein and the K. pneumoniae nif A protein in vivo. The DNA sequence of the R. meliloti nif H promoter has been compared with sequences of other promoters under the transcriptional control of gln G and DNA sequence homology was found in the -10 to -22 region (60). This suggests that the transcriptional control of nif genes in some Rhizobium species may be similar to that found in K. pneumoniae.

Expression of the nif and symbiotic genes in R. japonicum may be regulated in a different manner. RNA polymerase from both forms of R. japonicum appear to recognize the nif DK promoter in vitro. This suggests that transcriptional regulation of the nif genes may be controlled by a repressor which would block transcription of these genes in the vegetative state.

One approach which could be used to analyze this question would involve the use of an expression vector. The promoter for the nif DK operon could be fused to the carboxyl-terminus of β-galactosidase in a wide host-range plasmid. This plasmid would be used to transform R. japonicum bacteria which had been mutagenized with Tn 5. Mutants which expressed β-galactosidase under atmospheric $O_2$ tensions would be good candidates for cells which no longer had a functional nif repressor. Purification of such a protein could be accomplished by assaying for the inhibition of nif DK transcription using run-off transcription analysis or by selectively binding the repressor to cloned nif promoter DNA sequences coupled to agarose using DNA-agarose chromatography.

If a nif-specific repressor was purified from R. japonicum, it would be necessary to analyze the DNA sequences required for promoter binding.

It would also be of interest to characterize whether the repressor was specific for all or just a subset of *nif* and other symbiotic genes. This could be done by analyzing the transcription patterns of cloned *nif* genes *in vitro*. The characterization of RNA polymerase from *R. japonicum* presented here using both cloned *nif* genes and the well defined T7 DNA templates should be useful in this further analysis of the transcriptional regulation of nitrogen fixation in the *Rhizobium*-legume symbiosis.

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