THE 3'-TERMINI OF 16S RIBOSOMAL RNA FROM VEGETATIVE AND SPORULATING *MYXOCOCCUS XANTHUS*

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To my mother, Vida Dudzinski, and to my wife, Diane.

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ABSTRACT

The possibility that ribosomal RNA, synthesized during sporulation of Myxococcus xanthus, could represent a different type, which conferred upon ribosomes a greater affinity for spore-specific mRNA, was investigated. The binding of ribosomes to initiation sites in mRNA is thought to depend upon the sequence of bases at the 3'-end of 16S RNA in 30S ribosomal subunits (Shine and Dalgarno, 1975). Consequently, sequence analysis of the 3'-end of 16S RNA isolated from vegetative and sporulating M. xanthus cells was undertaken. The sequence, $_{HO}$U-U-U-U-G-U-U-U-U was found in both cases. The finding argues against regulation of protein synthesis during sporulation by a specific class of ribosomes.

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LIST OF ABBREVIATIONS

A	-adenosine
C	-cytidine
DEAE-cellulose	-diethylaminoethyl cellulose
DEAE Sephadex	-diethylaminoethyl Sephadex
DNA	-deoxyribonucleic acid
EDTA	-ethylenediamine tetraacetate
G	-guanosine
Isoniazid	-isonicotinic acid hydrazide
mRNA	-messenger ribonucleic acid
RCF	-relative centrifugal force
RNA	-ribonucleic acid
rRNA	-ribosomal ribonucleic acid
SDS	-sodium-dodecyl sulphate
tRNA	-transfer ribonucleic acid
U	-uridine

"It may be said that natural selection is daily and hourly scrutinising, throughout the world, every variation, even the slightest; rejecting that which is bad, preserving and adding up all that is good; silently and insensibly working, whenever and wherever opportunity offers, at the organic and inorganic conditions of life. We see nothing of these slow changes in progress, until the hand of time has marked the long lapses of ages, and then so imperfect is our view into long past geological ages, that we only see that the forms of life are now different from what they formerly were."

- Charles Darwin

INTRODUCTION

The early experiments of Lodish (1970) on bacterial ribosomes led to the conclusion that the 30S subunit was responsible for specificity of initiation of protein synthesis on polycistronic phage RNA. Subsequent experiments of Nomura et al., (1974) identified protein S12 and the 16S rRNA as the two most important elements within the 30S subunit in conferring specificity. Shine and Dalgarno (1974, 1975) extended this work by offering an explanation for ribosomal cistron specificity in terms of extensive base pair formation between the 5'-end of the polycistronic mRNA molecule and the 3'-end of 16S ribosomal RNA. The role of initiation factors, and of protein S12 would be to stabilise the complex formed between the messenger RNA molecule and the 30S subunit.

Although control of gene expression in most differentiating systems appears to be carried out chiefly at the level of transcription (Lodish, 1976) the work of Shine and Dalgarno offered the attractive possibility of control of gene expression at the level of translation, through cistron specificity on polygenic mRNA, exhibited by the 30S subunit of ribosomal RNA. This type of control of gene expression presents interesting possibilities in the case of morphogenesis of the fruiting myxobacteria. Through changes in the nucleotide sequence within the 3'-end of 16S rRNA, and by shifts in the protein population of this subunit, the ribosomes may efficiently select sporulation-specific mRNA.

The observation made by Dworkin (1964) that cells of Myxococcus xanthus, grown in a dispersed state in liquid culture, could be induced by 0.5 M glycerol to undertake rapid and synchronous sporulation has offered

a unique opportunity to test this possibility of change within the 16S ribosomal RNA.

HISTORICAL

The fruiting myxobacteria, a group of unusual procaryotes with complex life cycles were first described by Roland Thaxter in 1892. In a paper published in the Botanical Gazette in December of that year, Thaxter (1892) gave the following description:

"A few years since, while collecting fungi at Kittery and in several other localities in New England and the southern states, the writer's attention was attracted by bright orange-coloured growths occurring upon decaying wood, fungi and similar substances.....".

It was not until later observations of the complete life cycle of the organisms was made that their true bacterial nature became apparent (Thaxter, 1892). It was because of the complexity of this life cycle that Thaxter proposed a new order, Myxobacterales, be created within the Class Schizomycetes. It is within this order that the fruiting bacteria continue to be classified in Bergey's Manual of Determinative Bacteriology.

The myxobacteria are ubiquitous in nature, and are commonly found in soil, water and decaying plant and animal material (McCurdy, 1974). The order is defined according to Dworkin, (1972) by three principal features: (a) gliding motility on solid surfaces, (b) presence of a life cycle, and (c) the ability of the organisms to hydrolyse insoluble macromolecules.

The order is subdivided into four families on the basis of morphological criteria which include cell shape, microcyst formation, microcyst morphology, and finally, by the ability of the organisms to

elaborate sporangia. The four families include Polyangiaceae, Archangiaceae, Myxococcaceae, and Cystobacteraceae. The first family, Polyangiaceae is distinguished from the other families by the absence of microcysts and by the morphology of vegetative cells which are of uniform diameter, with blunt, rounded ends. The remaining three families are distinct in that they produce microcysts and by the fact that the vegetative cells are tapered. The morphological criteria which separate the other three families include the shape of the microcysts and the presence or absence of sporangia. The family Myxococcaceae elaborate spherical microcysts while in the Archangeaceae and Cystobacteraceae, the microcysts are rod-shaped. Finally, the families Archangiaceae and Cystobacteraceae are distinguished by the presence of a sporangium in the latter, and by its absence in the former.

Myxococcus xanthus, classified within the family Myxococcaceae are slender rods with tapering ends. The microcysts are spherical and not enclosed within a sporangium. The nucleus of vegetative cells of M. xanthus is typically procaryotic, with a mesosome-nucleoid complex commonly encountered in gram-negative cells. However, the unusual aspect is the distribution of total cell DNA between two nuclei which replicate sequentially (Dworkin, 1972). The size of the chromosome is 4.9×10^9 daltons (Zusman et al., 1968). In addition to this difference, the peptidoglycan of the cell wall is composed of overlapping plates rather than being a continuous bag-shaped macromolecule generally found in the eubacteria (Dworkin et al., 1968). The cells contain typical 70S bacterial ribosomes, and store polysaccharide and polyphosphate granules (Voelz, 1967).

The nutritional requirements for vegetative growth of M. xanthus were defined largely through the work of Dworkin (1962) with a strain capable of growing in a dispersed state in liquid medium. M. xanthus grows readily on defined medium containing 17 amino acids and several salts (Dworkin, 1962). No stimulation of growth was found with the addition of organic materials, with the exception of glycogen (Dworkin, 1962). In addition, $MgSO_4$ (around 0.1%) was required to sustain growth (Dworkin, 1962). When cultured on complex or defined media the cells divide by binary transverse fission, and grow aerobically with a generation time of 200-250 minutes (Dworkin et al., 1975).

By far the greatest element of uniqueness in this order is the complex life cycle that the organisms display; one in which individual cells interact socially to form fruiting bodies, within which certain individuals differentiate to form myxospores. In view of this social behaviour, the myxobacteria offer a superb opportunity to study the molecular mechanisms underlying cellular differentiation and the intricate mechanisms of cell interactions.

Recently, much attention has been focused on cellular interactions during aggregation and differentiation of the slime moulds (Newell et al., 1971; McMahon et al., 1973), eucaryotic organisms which the myxobacteria closely resemble; a striking example of evolutionary convergence. Research in this field would provide better insight into the processes that define normal tissue differentiation (Dworkin and Wireman, 1975), as well as abnormal, e.g., cancer.

The shift from growth to development is easily brought about in Myxococcus xanthus growing on solid media by starvation or deprivation

of specific amino acids (Dworkin, 1963). Under these conditions the cells migrate to aggregation centres. The precise nature of the response is not known, but the aggregation of cells towards a specific centre is promoted by cAMP and other adenine nucleotides (Lev, 1974). It has been observed that cultures of fruiting M. xanthus freely produce cAMP (Parish et al., 1976).

Fruiting body formation is the next step in the life cycle of these microbes. The structure of the fruiting body varies with the species. Certain members of the genus Myxococcus elaborate a fruiting body which is nothing more than an elevated mound of cells embedded in slime (Dworkin and Wireman, 1975). The next step up in structural complexity is exemplified by genera which elaborate fruiting bodies composed of a stalk that bears spores within a sporangium. The utlimate in fruiting body complexity is attained by genera such as Chondromyces and Stigmatella. In these, the spores are held in a series of cysts borne at the tips of branched stalks (Wireman and Dworkin, 1975) which are composed of hardened slime, within which are embedded a few cells (Reichenbach and Dworkin, 1969).

The function of the fruiting body and its relationship to spore formation remains an enigma. Dworkin (1972) suggested that formation of fruiting bodies may actually trigger the development of myxospores. Also, this structure may simply ensure that myxospores are formed in close proximity, which, in turn, ensures efficient germination; this last statement is reinforced by the observation of Ramsey and Dworkin (1968), that myxospores of M. xanthus will germinate in water only if their

population density is above a critical level. These explanations fail to account for the bright pigmentation and elaborate morphology of fruiting bodies such as those encountered in the genus Stigmatella. Dworkin (1972) has suggested that the colour and shape of the fruiting body may aid in recognition by insects that disseminate the mature spores.

Very little is known about the mechanism involved in the construction of fruiting bodies. Although various workers have attempted electron microscopic (Voelz et al., 1969; McCurdy, 1969) and time-lapse photomicrographic studies (Reichenbach, 1965), the details of cellular movements and cell physiology remain an enigma. It is possible that fruiting body formation involves the production, in an oriented fashion, of polysaccharides and lectins.

When the process of fruiting body formation is completed, further differentiation of certain cells into myxospores takes place. In M. xanthus this involves the conversion of a rod, measuring 5.0 μm x 0.7 μm into a round myxospore about 2 μm in diameter (Dworkin, 1972). At the same time, the differentiating cell acquires a capsule, optical refractility and increased resistance to heat, ultraviolet light, dessication and physical disruption (Dworkin, 1972).

The observation initially made by Dworkin and Gibson (1964), that it is possible to bypass the normal route of development from vegetative to spore stage, considerably simplified the study of differentiation in the myxobacteria. Sporulation, in the absence of fruiting body formation, may be brought about by adding glycerol (final concentration 0.5 M) to a culture of vegetative cells. A number of other compounds can induce spore formation, the chief among these being alcohols such as

threitol, erythrietol, isopropanol and phenethyl alcohol (Sadler and Dworkin, 1966). Dimethylsulfoxide is also an inducer of spore formation (Bacon and Rosenberg, 1967). Rosenberg et al., (1970), while studying the effects of amino acid deprivation on RNA synthesis, observed that methionine deprivation led to myxospore induction. Putrescine and feedback inhibitors of methionine synthesis had the same effect, while spermidine had an inhibitory effect on spore formation (Rosenberg et al., 1970).

When treated with agents that trigger spore formation the cells undergo stoichiometric conversion to spores in approximately 120 minutes (Dworkin et al., 1975). The mechanism by which induction takes place is not known. Studies with radioactively-labelled inducers demonstrate that transport across the membrane or intracellular metabolism of inducers was not necessary (Sadler and Dworkin, 1966). This strongly suggests that the inducers act at the level of the cell membrane by modifying or combining with specific membrane receptors; these changes being relayed to the gene loci responsible for spore formation.

A mechanism that partially explains the induction of spore formation by methionine starvation has been proposed by Cohen and Raina (1967). They showed that D-adenosylmethionine is a direct precursor of spermidine, an observation that strongly suggests methionine starvation depletes the cell of spermidine, which, as mentioned above, is an antagonist of myxospore formation. Moreover, Cohen and Raina (1967) suggested that the polyamines may have a direct effect on the regulation of RNA synthesis. The other possible effects may be on protein synthesis by promoting the association of ribosomal subunits.

The conversion from vegetative cells to spores entails major changes in the metabolism and macromolecular composition of the cells. With the onset of sporulation a number of enzymes are induced including those of the glyoxylate pathway (Dworkin, 1972). Related to this, is the utilization of poly-β-hydroxybutyrate, a process requiring the glyoxylate cycle, during sporulation (Dworkin, 1972). Spore formation is also characterized by increased synthesis and deposition of N-acetylgalactosamine on the cell surface. The incorporation of N-acetylgalactosamine into the spore coats coincides with the acquisition of resistance to sonic disruption. The structure of peptidoglycan also undergoes a transition during morphogenesis. The myxospore peptidoglycan is a continuous highly cross-linked, bag-shaped macromolecule, containing covelently bound glucose (Johnson and White, 1972), unlike the plates of peptidoglycans in vegetative cells (Dworkin et al., 1975).

Rosenberg et al., (1967) have ascertained that no new DNA synthesis is initiated with the onset of spore induction, although synthesis in progress goes to completion. This event is coupled with a cessation of net RNA synthesis although RNA turnover continues for as late as 200 minutes after induction (Bacon and Rosenberg, 1967). Moreover, the data of Bacon and Rosenberg (1967) indicates that some of the RNA synthesized during this period was ribosomal RNA suggesting that new ribosomes may be synthesized during sporulation. The synthesis of new ribosomes may be indicative of an active role for ribosomes in the differentiation process. This possibility was examined by Foster and Parish (1973), who found differences in the protein composition between 30S, but not 50S, ribosomal subunits of myxospores and vegetative cells.

More specifically, a higher content of 30S proteins, S6 and S4, was found in myxospores than in vegetative cells and the reverse was true for the ribosomal protein, S7.

Although it is not known whether these proteins are analogous, in Escherichia coli, proteins S4 and S7 play an important role in the recognition of mRNA (Stoffler, 1974). Antibodies directed against E. coli 30S proteins, S4, S7, S15 and S16, are strong inhibitors of T4 phage mRNA-directed protein synthesis, and also of λ phage DNA-directed β-galactosidase synthesis (Stoffler, 1974). Antibodies generated against protein S6 inhibit the binding of initiator tRNA (Traut et al., 1974). All three protein species (S4, S6, S7) are bound to 16S rRNA (Stoffler, 1974).

Changes in ribosomal structure also take place during development of Bacillus subtilis. Ribosomes of this organism lose the ability to bind the antibiotic, fusidic acid, at the time of asymmetric formation of prespore septa (Fortnagel and Freese, 1977). Fortnagel and Freese (1977) claim that the inability of fusidic acid to interact with resistant ribosomes stems from a change in the ribosome-EF-G complex. The change in bacterial ribosomes has also been detected, by means of SDS-gel electrophoresis (Fortnagel et al., 1973).

There are several ways by which a cell may regulate the synthesis of proteins during development. These include preferential gene replication, controls operative at the level of transcription, attachment of the transcript to ribosomes, rate of degradation of the transcript and rate of processing of a precursor mRNA into a translatable form (Lodish, 1976). In bacterial cells, gene expression is, for

the most part, regulated at the level of transcription (Lodish, 1976). In Escherichia coli, there exists a direct relationship between the amount of transcripts generated for any given operon, and the rate of synthesis of polypeptides from the transcripts. This holds true for the lactose (Varmus et al., 1970), arabinose (Schleif, 1971) and galactose (Miller, 1971) operons.

There are indications that a change in the RNA polymerase may take place during development of Myxococcus xanthus. The mature myxospores of M. xanthus are resistant to infection by phage MX-1, while vegetative cells are susceptible (Dworkin, 1972). When vegetative cells are infected at the onset of spore induction, the developmental pathway is aborted, and the cells undergo lysis. However, a small percentage of cells are "cryptically" infected, before the phage receptors become altered or masked. The infected cells proceed along their programmed path and produce "normal" myxospores, but upon germination, some cells lyse, releasing mature phages. This observation led to the proposal that the phage maturation within cryptically-infected cells was prevented by a change in RNA polymerase that occurred 20 minutes after induction of sporulation, such that it was unable to transcribe phage DNA (Dworkin, 1972). Losick et al., (1969) have also reported a change in the template specificity of RNA polymerase during sporulation of Bacillus subtilis. The change was characterized by the inability of RNA polymerase of sporulating cells to transcribe phage DNA, while RNA polymerase from vegetative cells was fully active with this template (Losick et al., 1969).

Cells may regulate translation by controlling the availability of initiator transfer RNA and 30S ribosomal subunits, or by sequestering

mRNA in ribonuclease-resistant nucleoprotein complexes. This last method has been observed in the case of sea-urchin and amphibian oocytes (Lodish, 1976). Evidence for this type of long-lived mRNA in procaryotes is indirect (Dworkin, 1972). In Myxococcus xanthus, inhibition of RNA synthesis by streptomycin or actinomycin D in myxospores, at dosages that inhibited vegetative growth did not inhibit germination (Dworkin, 1972). This observation hinted strongly at the presence of mRNA that was pre-formed at the time of spore formation, which could be activated during germination. However, attempts to isolate such a message have been unsuccessful (Dworkin, 1972).

One point where cells might exert selective control over mRNA translation could be by altering the specificity of initiation factors. In E. coli systems, the recognition of natural messages by ribosomes, such as T_4 phage mRNA and f2 phage RNA, requires IF-3. Some researchers in this field claim to have isolated several forms of this initiation factor, each exhibiting different cistron specificities with polygenic mRNA (Pollack et al., 1970; Grunberg-Manago et al., 1971). Lee-Huang and Ochoa (1971) isolated two types of IF-3 from E. coli; one which enhanced translation of MS2 phage RNA and E. coli mRNA but not late T_4 phage mRNA, while the reverse was true for the other form. Several groups have recently reported the isolation of protein factors which alter the specificity of IF-3 for mRNA (Grover et al., 1972; Lee-Huang et al., 1972). There is considerable ambiguity in this area, as other groups could identify only one species of IF-3 in Escherichia coli, which exhibited no preference for any type of mRNA (Spermulli et al., 1974; Schiff et al., 1974). It is possible that the different species

of IF-3 were generated by proteolytic cleavage of a single species during, or prior to, isolation (Lodish, 1976).

Evidence exists, on the other hand, that ribosomes exhibit specificity for mRNA and that this selectivity primarily resides in the 30S ribosomal subunit. Ribosomes from E. coli initiated translation on all three cistrons of bacteriophage f2 RNA while Bacillus ribosomes were unable to (Leffler et al., 1973, 1974). The components required for protein synthesis were isolated from the two bacterial species and interchanged to construct "hybrid" protein synthesizing systems. It was found that the source of initiation factors, tRNA and 50S subunits did not alter the ability of ribosomes in binding to phage f2 RNA. The ability of the hybrid system to initiate synthesis was determined solely by the source of the 30S subunit (Leffler et al., 1973, 1974; Lodish, 1970). Nomura et al., (1974) extended this work, to define the components of the 30S subunits which were responsible for the specificity exhibited by ribosomes. They constructed 30S subunits of E. coli in which the 16S RNA and/or specific 30S proteins were replaced by their analogues from B. stearothermophilus. The hybrid subunit was tested in a protein-synthesizing system programmed with RNA obtained from E. coli phage R17, a RNA on which E. coli, but not B. stearothermophilus, 30S subunits can initiate translation. When the 30S protein, S12, or 16S RNA in the hybrid subunit was derived from B. stearothermophilus, translation was decreased by 50%, as compared to that programmed by synthetic polyuridylic acid, and 85% when both components were replaced. Goldberg et al., (1974) confirmed the above observation with respect to protein S12, but did not study the involvement of 16S ribosomal RNA.

The experiments of Konisky and Nomura (1967) led to additional evidence for the role of 16S RNA in initiation of protein synthesis. It was found that ribosomes from E. coli cells treated with colicin E3 had very little activity in in vitro polypeptide synthesis. The altered ribosomal component that resulted in loss of activity was identified by Bowman et al., (1971) to be 16S rRNA. The inactive 16S ribosomal RNA was missing a 50-nucleotide fragment from the 3'-end (Bowman et al., 1971). Finally, the work of Helser et al., (1971), and Dahlberg et al., (1973), with streptomycin and kasugamycin, two inhibitors of polypeptide chain initiation, indicated that their site of activity is the 3'-end of 16S rRNA.

To explain the specificity of bacterial ribosomes for mRNA, Shine and Dalgarno (1975) offered a hypothesis based on base complementarity between the 3'-end of the 16S ribosomal RNA and the 5'-end of mRNA. The method of Hunt (1970) was employed to establish the sequence $_{HO}$A-U-U-C-C-U-C-C-A for the 3'-end of 16S RNA from Escherichia coli. The sequence was then compared to the 5'-ends of several RNA phages and E. coli mRNA to determine any base-pair complementarity. The phage RNA examined contained either a part of, or the complete sequence (5') $_{p}$A-G-G-A-G-G-U, roughly ten base pairs removed on the 5'-side of the initiation codon, AUG (Table 1). Although, in some cases, as few as three bases are complementary to 16S rRNA, this may be sufficient to ensure specificity in binding 30S subunits to mRNA (Table 1). Moreover, the comparison shows that seven base pairs can be formed between the 16S rRNA and the initiation site of phage R17 maturation protein A; while only four or five base pairs can be formed between the rRNA and the

TABLE 1.

INITIATION SEQUENCES RECOGNIZED BY *Escherichia coli* RIBOSOMES[a,b]

mRNA	Ribosome binding site
R17 A	GAU UCC UAG GAG GUU UGA CCU AUG CGA GCU UUU AGU G
Qβ A	UCA CUG AGU AUA AGA GGA CAU AUG CCU AAA UUA CCG CGU
R17 coat	CC UCA ACC GCG GUU UGA AGC AUG GCU UCU AAC UUU
Qβ coat	AAA CUU UCG GUC AAU UUG AUC AUG GCA AAA UUA GAG ACU
f2 coat	CC UCA ACCG(A,G)GUU UGA AGC AUG GCU UCC AAC UUU ACU
R17 replicase	AA ACA UGA GGA UUA CCC AUG UCG AAG ACA ACA AAG
Qβ replicase	AG UAA CUA AGG AUG AAA UGC AUG UCU AAG ACA G
f1 coat	UUU AAU GGA AAC UUC CUC AUG AAA AAG UCU UU
f1 gene 5	A AGG UAA UUC ACA AUG AUU AAA GUU GAA AU
f1 gene ?	A AAA AAG GUA AUU CAA AUG AAA UU
T7 *in vitro*	AAC AUG AGG UAA CAC CAA AUG AUU UUC ACU AAA GAG
T7 gene 0.3	ACG AGG UAA CAC AAG AUG GCU AUG
λ P_R	pppAUG UAC UAA GGA GGU UGU AUG GAA CAA CGC
φX174 spike (DNA)	TTT CTG CTT AGG AGT TTA ATC ATG TTT CAG ACT TTT ATT
trp leader	CAC GUA AAA AGG GUA UCG ACA AUG AAA GCA AUU UUC GUG
*trp*E	GAA CAA AAU UAG AGA AUA ACA AUG CAA ACA CAA AAA CCG
*trp*A	GAA AGC ACG AGG GGA AAU CUG AUG GAA CGC UAC GAA UCU
*lac*Z	AAU UUC ACA CAG GAA ACA GCU AUG ACC AUG AUU ACG GAU
*lac*I pppG GAA GAG	AGU CAA UUC AGG GUG AAU GUG GUG AAA CCA GUA ACG
*gal*E	AUA AGC CUA AUG GAG CGA AUU AUG AGA GUU CUG GUU ACC
16 S RNA 3' end	mαA U U C C U C C A C U A G

[a] Taken from Gros et al., (1977)

[b] Underlining indicates contiguous bases complementary to the 3'-terminal oligonucleotide of E. coli 16S RNA. Dots indicate G-U base pairs. Gaps appear where a bulge in the rRNA strand is required to provide the indicated complementarity.

other R17 initiation sites. From this observation, it would be expected that, in vitro, more maturation protein would be synthesized in the presence of the A-protein RNA fragment, than in the presence of other RNA fragments. This is confirmed by the observation of a much greater and more efficient interaction of E. coli ribosomes with the isolated A-protein RNA fragment, than with the coat and replicase RNA fragments (Steitz, 1973). While this contrasts with the observation that with intact phage RNA the efficiency of translation of the A-protein is considerably decreased, the discrepancy is explained by the secondary structure in phage RNA which impedes the binding of 30S ribosomal subunits (Steitz, 1973). The base sequences for several mRNA fragments protected by ribosomes have been determined (Steitz, 1969; Hindley et al., 1969). While several fragments exhibit considerable secondary structure (Steitz, 1969; Hindley et al., 1969), others do not (Steitz, 1973; Maizels, 1974; Weber et al., 1972). For this reason, it would seem highly unlikely that secondary structure plays a role in the ribosome recognition process.

Steitz and Jakes (1975) provided compelling evidence for base-pair formation between the 5'-end of mRNA and 16S ribosomal RNA. Radioactive initiator fragments from phage R17 RNA were allowed to form initiation complexes with E. coli ribosomes. The complexes formed were treated with colicin E3, and disassembled by exposure to 1% SDS. Electrophoresis on polyacrylamide gels revealed a complex between the 50-nucleotide colicin fragment from rRNA and the 30-nucleotide phage fragment. This complex did not appear if the colicin treatment was omitted.

Treatment with aurintricarboxylic acid, which inhibits mRNA binding to ribosomes, was found to lower the amount of complex formed (Steitz and Jakes, 1975).

While the Shine and Dalgarno hypothesis predicts a positive correlation between the number of base-pairs formed between mRNA and the 3'-end of 16S rRNA, specificity of initiation should not be affected by the source of the ribosomes, provided the criterion of complementarity is satisfied. This possibility was tested by several workers, with ribosomes isolated from sources other than Escherichia coli. Szer et al., (1970), demonstrated that all three cistrons of MS2 phage RNA were translated with the equal fidelity by E. coli and Pseudomonas aeruginosa ribosomes. A comparison of E. coli and Pseudomonas 16S rRNA revealed identical 3'-end for a length of seven nucleotides (Table 2). In contrast to the P. aeruginosa 16S RNA, the 3'-ends of B. stearothermophilus and E. coli rRNA differ significantly (Table 2). However, about 4 complementary base pairs can be formed between the 3'-end of 16S RNA of B. stearothermophilus and the A-protein and replicase ribosome binding sites of phage R17 RNA, while only one to two base pairs are complementary to the coat protein initiation site (Tables 1 and 2). This observation is in agreement with the experiments of Lodish (1971), who detected translation of A-protein and replicase segments of R17 RNA but not coat protein synthesis by Bacillus ribosomes.

Steitz (1973), working with B. stearothermophilus ribosomes demonstrated that mRNA recognition can take place in the absence of protein synthesis. It was found that Bacillus ribosomes bound only a

TABLE 2.

3'-TERMINAL SEQUENCES OF 16S rRNA[a,b]

Escherichia coli	GAUCACCUCCUUA$_{OH}$
Pseudomonas aeruginosa	G(X)$_2$YCUCUCCUU(A)$_{OH}$[c]
Bacillus stearothermophilus	G(X)$_5$YUCCUUUCU(A)$_{OH}$[c]
B. stearothermophilus	GAUCACCUCCUUUCUA$_{OH}$[c]
B. subtilis	G(X)$_7$YCUUUCU$_{OH}$
Caulobacter crescentis (ATCC 15252)	G(X)$_3$YUCCUUUCU$_{OH}$

[a] From Shine and Dalgarno (1975)

[b] X represents any nucleoside other than guanosine; Y = pyrimidine nucleoside.

[c] The variable presence of the 3'-terminal adenosine in 16S RNA is found in a variety of bacteria and depends on the culture conditions.

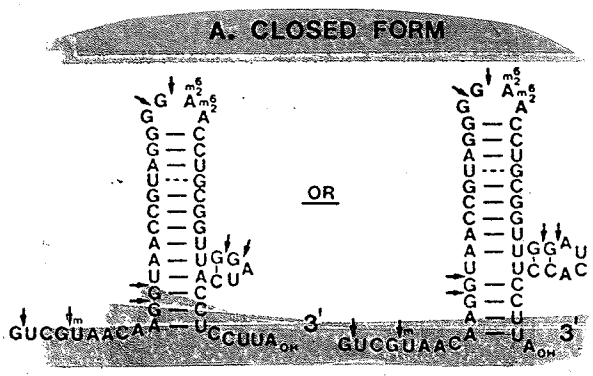
single site of phage QB RNA at 65° C, which did not correspond to any of the three normal initiator regions. Although the fragment was of a similar size to the A-protein cistron of R17 RNA, it contained no initiation triplet. The fragment was found to be rich in purine nucleotides, and had the composition G(AAAG, AG, G,G,G)A in a position similar to that of (5')A-G-G-A-G-G-U(3') in the R17 A-protein site (Steitz, 1973). These fragments exhibit complementarity to the 3'-end of B. stearothermophilus 16S RNA, (5')A-G-G-A-A-A-G-A(3'). Sprague et al., (1977) determined the exact sequence of the purine rich segment of QB RNA that was bound by the B. stearothermophilus ribosomes and confirmed the existence of extensive complementarity between Bacillus 16S rRNA and the phage RNA fragment. Gros et al., (1977) suggest that the failure of B. stearothermophilus ribosomes to recognize the QB RNA fragment at lower temperatures resulted from extensive secondary structure of QB RNA under these conditions.

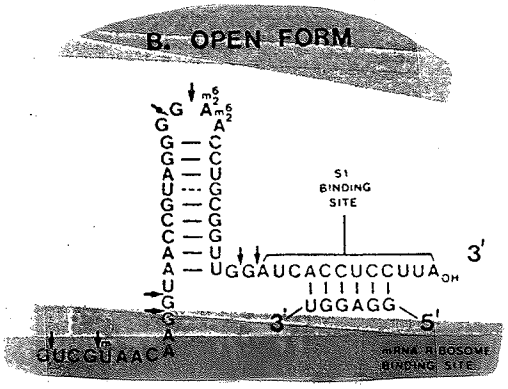
Several workers have proposed a secondary structure for E. coli 16S rRNA, in which the RNA is folded in a series of hairpin loops linked by short single-stranded regions (Ehresmann et al., 1975; Cox et al., 1973; Fellner et al., 1972). Ehresmann et al., (1975) proposed the existence of a hairpin loop at the 3'-end of the 16S rRNA. Dahlberg and Dahlberg (1975) suggested an alternate structure for the 3'-end, with more extensive base pairing (Figure 1). The formation of a hairpin loop at the 3'-end of 16S RNA would interfere with base pair formation between the ribosomal RNA and mRNA. Recently, however, Dahlberg and Dahlberg (1975) have presented evidence that the ribosomal protein, S1,

FIGURE 1

Proposed structures of E3 RNA in the presence and absence of S1. (A) Structure proposed by Ehresmann et al., (1975) (left) and an alternate structure proposed by Dahlberg and Dahlberg (1975), in which there is more pairing of the 3'-terminal bases (right). (B) Structure which has the 3'-terminal bases in an open form. This structure, which may exist with or without S1, unfolds the A-C-C-U-C-C sequence, which is complementary to ribosome binding sites of several mRNAs. Arrows mark the sites susceptible to cleavage by RNase T1.

1.


A. CLOSED FORM
OR
GUCGUAACA
3'
GUCGUAACA
3'


B. OPEN FORM
S1 BINDING SITE
3'
UGGAUCACCUCCUUA
UGGAGG
3'
5'
GUCGUAAC
mRNA RIBOSOME BINDING SITE

binds to the 3'-end of 16S rRNA in such a manner that the hairpin loop at the 3'-end of the molecule is abolished, making this site available for binding to the initiator regions of mRNA (Fig. 1).

In view of the observed changes in ribosomal structure during morphogenesis of Myxococcus xanthus, the hypothesis of Shine and Dalgarno (1975), offers an attractive possibility that protein synthesis during morphogenesis of this microbe may be modulated by changes in the base sequence at the 3'-end of 16S ribosomal RNA. This change would allow ribosomes to actively select mRNA molecules required for the synthesis of sporulation-specific proteins. The ability of Myxococcus xanthus to undergo rapid transition from vegetative to spore form, upon induction by glycerol, offered an excellent opportunity to test this idea.

MATERIALS AND METHODS

Materials

Most of the reagents and chemicals were obtained from Fisher Scientific Company. Sodium-4-aminosalicylate, isonicotinic acid hydrazide, ribonuclease-free alkaline phosphatase, ribonuclease T1, pancreatic ribonuclease, Tris base, and nucleosides were obtained from Sigma Chemical Company. (G-^{3}H)-isonicotinic acid hydrazide was from Amersham-Searle Corporation; ribonuclease-free sucrose from Schwarz-Mann; E. coli transfer RNA from Grand Island Biological Company; and acrylamide and N,N'-methylenebisacrylamide from Eastman.

Organism

Myxococcus xanthus MD 1, formerly strain FB was obtained from Dr. M. Dworkin, University of Minnesota. The organism was routinely grown in 2% (w/v) Difco casitone containing 0.01 M $MgSO_4$ i.e., CM medium, at 32°C with vigorous shaking in a gyrorotatory or reciprocating shaking water bath (New Brunswick Scientific Company) to provide aeration. For storage, log phase cultures were made 0.5M with sterile glycerol, distributed in 1.0 ml volumes in sterile glass vials, then stored at -70°C. Such stocks remained viable for at least one year. The cultures were checked periodically for competence in forming fruiting bodies by streaking on 2% (w/v) Difco agar plates and observing the appearance of fruiting structures after 4-5 days incubation at 32°C.

Growth

Stock cultures were inoculated into 40 ml of CM medium in

250 ml Erlenmeyer flasks and grown to mid-log phase of growth in a shaking water bath maintained at 32°C. This corresponded to 250 Klett units when readings were made with a red filter (see Results). Each 40 ml culture was seeded into 400 ml of CM medium in 2 l Erlenmeyer flasks which were shaken at 160 rpm in a Model G53 gyratory shaker (New Brunswick Scientific Company. In this case, incubation was carried out at 28°C, the temperature at which the incubation room containing the shaking apparatus was maintained. When culture density reached 250 Klett units (red filter), the cells were centrifuged at 4°C in a RC 2B refrigerated centrifuge (Sorvall) with a GS 3 rotor (capacity, 2700 ml) at 12,000 RCF for 20 min. Approximately 2 g (wet weight) of cells were obtained from 400 ml of medium.

To induce sporulation, vegetative cells were grown as above, but when the culture density reached 250 Klett units (red filter), the medium was made to 0.5 M glycerol by addition of sterilized 6.8 M glycerol. Within 200 minutes, greater than 95% of the cells had been converted to myxospores, as judged by their morphological appearance when bacterial counts were made with a Petroff-Hausser counting chamber (Hausser Scientific). Cells were harvested 60 minutes after glycerol addition as described above for vegetative cells. In other cases, incubation was continued for 120 minutes after addition of glycerol, at which time the medium was first made 0.1% with SDS for 15 minutes before harvesting. SDS lyses all vegetative cells so that the pellets consisted primarily of myxospores. When harvested cells could not be used for RNA extraction immediately, they were stored frozen at -70°C.

RNA Extraction

The unit of measurement for RNA used in this thesis is "A_{260} unit", which is the amount of RNA that gives an absorbance of 1.0 at 260 nm in a cuvette with 1.0 cm path length. It was assumed that 1 mg of ribosomal RNA was equivalent to 25 A_{260} units (Cox, 1970). RNA was isolated from cells following a modification of the method of Kirby (1965). The composition of the two solutions used in the extraction process is given below.

Solution A: 6.0 g sodium-4-aminosalicylate
1.0 g sodium chloride
0.1 g sodium dodecyl sulfate
0.08 g disodium ethylenediaminetetraacetic acid
3.0 ml phenol mixture (see below)

The above components were dissolved in distilled water to make a total of 1 l.

Phenol Mixture: 500 g phenol crystals
1.0 g 8-hydroxyquinoline
70 ml m-cresol
55 ml distilled water

Cells that were harvested during sporulation were ground with alumina at a cell to alumina ratio of 1:1.5 (w/w) with a mortar and pestle at 0°C. Each gram of cell was then extracted with 7 volumes of solution A and centrifuged at 12,000 RCF for 5 minutes at 10°C. The supernatant was treated in the identical manner to the crude extract of

vegetative cells. The crude extract of vegetative cells was prepared by lysing cells by stirring the cell pellet with 7 volumes (w/v) of solution A at 10°C, followed by centrifugation at 12,000 RCF for 20 minutes at 10°C to remove any debris.

All following procedures were carried out at room temperature, except centrifugations, which were conducted at 10°C. This was necessary to prevent the precipitation at 4°C of SDS which was included to inhibit ribonuclease activity. The extract was stirred for 30 minutes with 0.5 volumes (v/v) of the phenol mixture, then centrifuged at 12,000 RCF for 20 minutes to effect phase separation. The top aqueous phase was carefully removed with a 50 ml plastic syringe fitted with glass tubing (bore size, 3mm), and the lower phenol phase was re-extracted with 0.5 volumes (v/v) solution A for 20 minutes. After centrifugation, as above, the aqueous phase was combined with that of the first extraction and cooled in ice. Two volumes (v/v) of ethanol (-20°C) were stirred into the combined aqueous extracts and the voluminous precipitate that formed after 2 h at -20°C was collected by centrifugation at 12,000 RCF for 15 minutes.

The pellet was extracted 3 times with 3.0 M sodium acetate, pH 6.0, containing 0.1% SDS (w/v) to remove contaminating polysaccharides and DNA, then dissolved in 0.1 M sodium acetate (pH 5.0) containing 0.1% SDS. To remove residual polysaccharides, the preparation was centrifuged for 30 minutes in a L2-65B Beckman ultracentrifuge at 25,000 rpm in a 50 Ti rotor (average RCF, 26,000). The supernatant containing 2,000 to 3,000 A_{260} units of RNA from 15 g wet weight of cells was the source for 16S ribosomal RNA. If not used immediately, the preparation was precipitated with 2 volumes of ethanol and stored at -20°C.

Isolation of 16S Ribosomal RNA

Two cycles of centrifugation through sucrose gradients were required in order to purify 16S RNA. The centrifugations were carried out in 5-20% (w/v) sucrose gradients in 0.1 M Na acetate, pH 5.0, containing 0.1% SDS (volume, 60 ml). Approximately 700 A_{260} units of RNA in 2 ml were layered on each gradient and centrifuged for 20-24 h in a SW 25.2 rotor (Beckman Instrument Company) at 15°C in an L2-65B Beckman ultracentrifuge. The gradients were emptied from the bottom with a polystaltic pump and 1.5 ml fractions were collected. A portion of each fraction was diluted with water and the absorbance at 260 nm was measured with a Beckman DU spectrophotometer. The fractions containing 16S RNA was recovered by precipitation with 2 volumes of ethanol at -20°C for 2 h, and dissolved in 0.1 M Na acetate, pH 5.0, containing 0.1% SDS, to a concentration of 350 A_{260} units/ml. One ml of this preparation was centrifuged through a second sucrose gradient and fractionated as described above. The pooled 16S RNA was precipitated with 2 volumes of ethanol and stored at -20°C.

Acrylamide Gel Electrophoresis

The purity and integrity of isolated 16S RNA was determined by polyacrylamide gel electrophoresis in a continuous buffer system described by Peacock and Dingman (1968). The running buffer consisted of 10.8 g Tris base, 0.93 g disodium EDTA, 5.5 g boric acid and 1.0 g SDS in 1 l distilled water. To make the gel, the following was dissolved in 100 ml of the running buffer: 2.5 g acrylamide, 0.15 g N,N'-methylenebisacrylamide, 0.075 g ammonium persulfate and 10 µl of N,N,N',N'-tetra-

methylenediamine. Gels were cast in glass tubes (inner diameter, 6 mm) and pre-run for 1 hr at 1 ma/tube before application of the sample. Approximately 0.5 A_{260} units of RNA in the running buffer was made 5% (w/v) with sucrose and layered on each gel with a trace of bromophenol blue which served as a marker for the front. Electrophoresis was conducted at 3 ma/tube for 2 h. After electrophoresis was complete, gels were removed from the tubes, fixed in 1% (v/v) acetic acid. Migration of RNA was determined by scanning the gel in a Joyce-Loebl U.V. scanner equipped with a chart recorder.

Periodate Oxidation of RNA

Ethanol-precipitated 16S RNA was dissolved in 0.1 M Na acetate (pH 5.0) to a concentration of 1-5 mg/ml. (25-125 A_{260} units/ml). The RNA was oxidized with a 100-fold molar excess of freshly-prepared 0.2 M sodium metaperiodate (pH 4-5) for 1 h at 22°C in the dark. Assuming a molecular weight of 560,000 (Kurland, 1960; Stanley and Bock, 1965), 1 mg of 16S RNA is equivalent to 1.8 nanomoles. The reaction was terminated by precipitation of the RNA with 2 volumes of ethanol at -20°C for 2 h. The precipitate, recovered by centrifugation, was redissolved in the original volume of 0.1 M Na acetate (pH 5.0). A small portion of the oxidized RNA was labelled with ^{3}H-isoniazid, while the remainder was subjected to stepwise degradation to remove the oxidized nucleoside at the 3'-terminus (see below).

Stepwise Degradation of RNA

The oxidized RNA was precipitated with 2 volumes of ethanol for 2 h at -20°C and the precipitate, recovered by centrifugation, was

dissolved in 0.33 M freshly-distilled aniline (adjusted to pH 5.0 with concentrated HCl) to give the original concentration. The procedure removes the 3'-oxidized nucleoside by a β-elimination reaction (Steinschneider and Fraenkel-Conrat, 1966). The reaction was carried out at 22°C for 4 h, and terminated by addition of 0.1 volumes (v/v) of 1.0 M Na acetate (pH 5.0) and 2 volumes of ethanol. After 2 h at -20°C, the precipitated RNA was recovered by centrifugation and dissolved in 0.02 M ammonium acetate (pH 7.0) and 1.0 mM $MgCl_2$ at double its original concentration (generally about 10 mg/ml). To remove the 3'-phosphate that resulted from elimination of the oxidized nucleoside, the RNA was incubated with 20 μg/ml of alkaline phosphatase for 45 minutes at 37°C. The mixture was cooled in ice, and the RNA precipitated with 2 volumes of ethanol at -20°C for 2 h. The precipitate was then dissolved in 0.1 M Na acetate (pH 5.0) to its original concentration (1-5 mg/ml). The sequential treatments with periodate, aniline and alkaline phosphatase cleaves the terminal nucleotide from 16S RNA to form a new 3'-OH terminus. By repeated cycles of treatments, successive nucleotides can be cleaved from the 3'-terminus in a stepwise manner. For sequencing, the procedure was repeated 7 to 8 times.

Labelling Oxidized RNA with ^{3}H-isonicotinic Acid Hydrazide (isoniazid)

The periodate-oxidized RNA (see above) was also labelled with ^{3}H-isoniazid (specific activity 0.8 Ci/mmol), according to the method of Hunt (1970). The oxidized RNA in 0.1 M Na acetate pH 5.0 was reacted with a 100-fold molar excess of ^{3}H-isoniazid for 18 h at 22°C. The labelled RNA was precipitated with 2 volumes of ethanol at -20°C for

2 h, dissolved in 0.01 M Na acetate, pH 5.0, containing 0.2 M NaCl then adsorbed to a 1 x 3 cm column of DEAE-cellulose (Whatman) equilibrated with the same buffer. Unreacted ^{3}H-isoniazid was washed from the column with 50 ml of the equilibrating buffer, then the labelled RNA was eluted with 4 ml of 2.0 M Na acetate, pH 5.0. The fractions were monitored for radioactivity by counting aliquots in Bray's (1960) scintillation fluid in a Beckman LS 230 scintillation counter. The RNA was precipitated with ethanol as above, and dissolved at a concentration of 1 mg/ml in 0.01 M Na/K phosphate buffer, pH 7.4.

Identification of 3'-Terminally-Labelled Nucleoside

The ^{3}H-labelled-RNA was digested with pancreatic ribonuclease (100 μg/mg RNA) and ribonuclease T1 (10 μg/mg RNA) at 22°C for 4 h. The enzymes catalyze the scission of phosphodiester bonds on the 3'-side of guanine, or pyrimidine nucleotides in RNA. Consequently, the labelled 3'-terminus would be released as a nucleoside or oligonucleotide ^{3}H-isonicotinoylhydrazone.

The identity of the labelled digestion product was determined by paper electrophoresis. Ten to 20 μl of digest was mixed individually with 10 to 20 μl of 4 unlabelled marker mononucleoside hydrazones (see later). The four mixtures were spotted separately on Whatman 3 mm paper and electrophoresed at 40 V/cm for 2.5 h in a Savant flat plate electrophoresis apparatus. The running buffer was 0.12 M Na formate, pH 3.0. After drying, the positions of marker nucleoside hydrazones and free isoniazid, (present in the marker solutions), were detected under utraviolet light, then the electropherogram was cut in 3 cm strips. Each

strip was cut into 2 x 3 cm pieces and assayed for radioactivity in a scintillation cocktail consisting of 0.5 g 1,4-bis-[2-(5-phenyloxazolyl)]-benzene, and 5.4 g 2,5-diphenyloxazole, dissolved in 285 triton X-100 and 715 ml toluene. (Shine and Dalgarno, 1974).

Preparation of Marker Nucleoside Isonicotinoyl Hydrazones

These were prepared according to the method of Hunt (1965). One ml volumes containing 4 μmoles adenosine, 5 μmoles guanosine, 10 μmoles cytidine or 10 μmoles uridine in distilled water were oxidized with 1.3 fold molar excess of 0.2 M freshly prepared sodium metaperiodate for 1 h at 25°C in the dark. The oxidized nucleosides were condensed with a 4-fold molar excess of isoniazid at 25°C for 16 h. The standards were stored at 4°C and were discarded after 1 week.

DEAE Chromatography

A modification of the chromatography procedure of Hunt (1970) and Shine and Dalgarno (1974) was followed to determine the chain length of oligonucleotide hydrazones released by ribonuclease T1 digestion of labelled RNA. Chromatography was carried out on columns (1 x 25 cm) of A-25 DEAE Sephadex (Pharmacia), equilibrated with 0.01 M sodium/potassium phosphate buffer (pH 7.4), made with 7.0 M charcoal-washed urea. Elution was with a 1200 ml linear gradient of 0.1 to 0.6 M Na Cl in 0.01 M Na/K phosphate buffer (pH 7.4) containing 7.0 M urea at a flow rate of 24 ml/hr. The absorbance at 260 nm of each column fraction (5.0 ml) was measured with a Gilson Model 3000 spectrophotometer attached to a Beckman DU monochronometer. For determination of radioactivity, 1 ml from each fraction was mixed with 10 ml of "Scintiverse" scintillation cocktail

(Fisher Scientific Co.) and counted in a Beckman LS-230 scintillation counter. All counts were corrected for background and quenching.

The sample containing ^{3}H-isonicotinic acid-labelled RNA and 1.0-2.0 mg carrier transfer RNA was digested with ribonuclease T1, and made 7.0 M with solid urea before application to the column. Digestion of tRNA was at 37°C for 2 h in 0.01 M Na/K phosphate buffer (pH 7.4) with 10 μg ribonuclease T1/mg RNA. For labelled RNA, digestion was carried out at 20°C for 3 h. Before and after digestion, the samples were briefly heated (90°C, 30 s), and rapidly cooled in ice to denature the RNA.

The elution positions of the various oligonucleotides generated by digestion was determined by including 0.5 mg adenosine, 3', 5'-cyclic adenosine monophosphate or 2'(3')-mixed adenosine monophosphate with the samples. Their elution positions, as noted by increased absorbance at 260 nm, identified the positions at which nucleosides, dinucleoside monophosphates, and trinucleoside diphosphates, respectively, were eluted from the column.

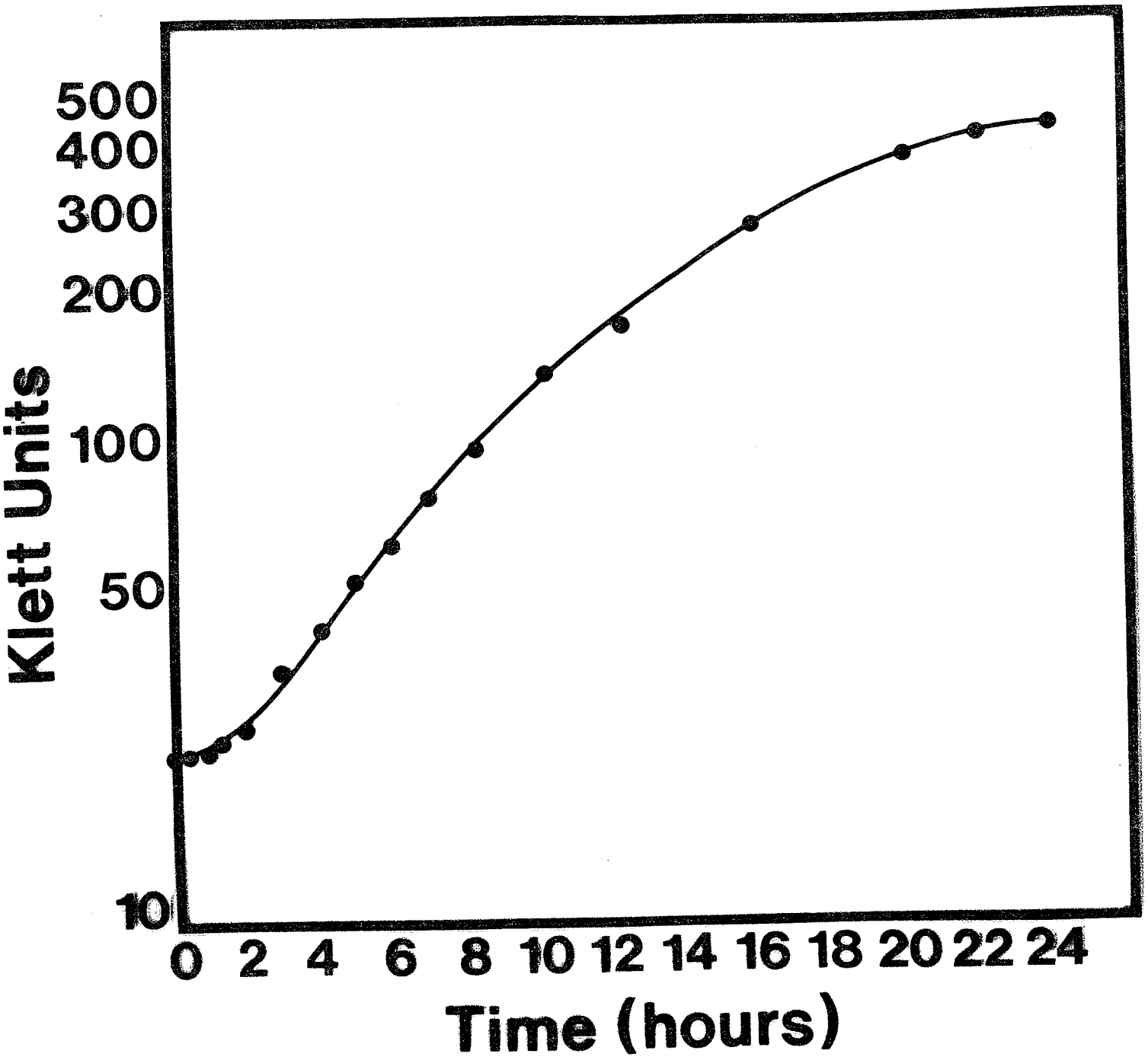
RESULTS

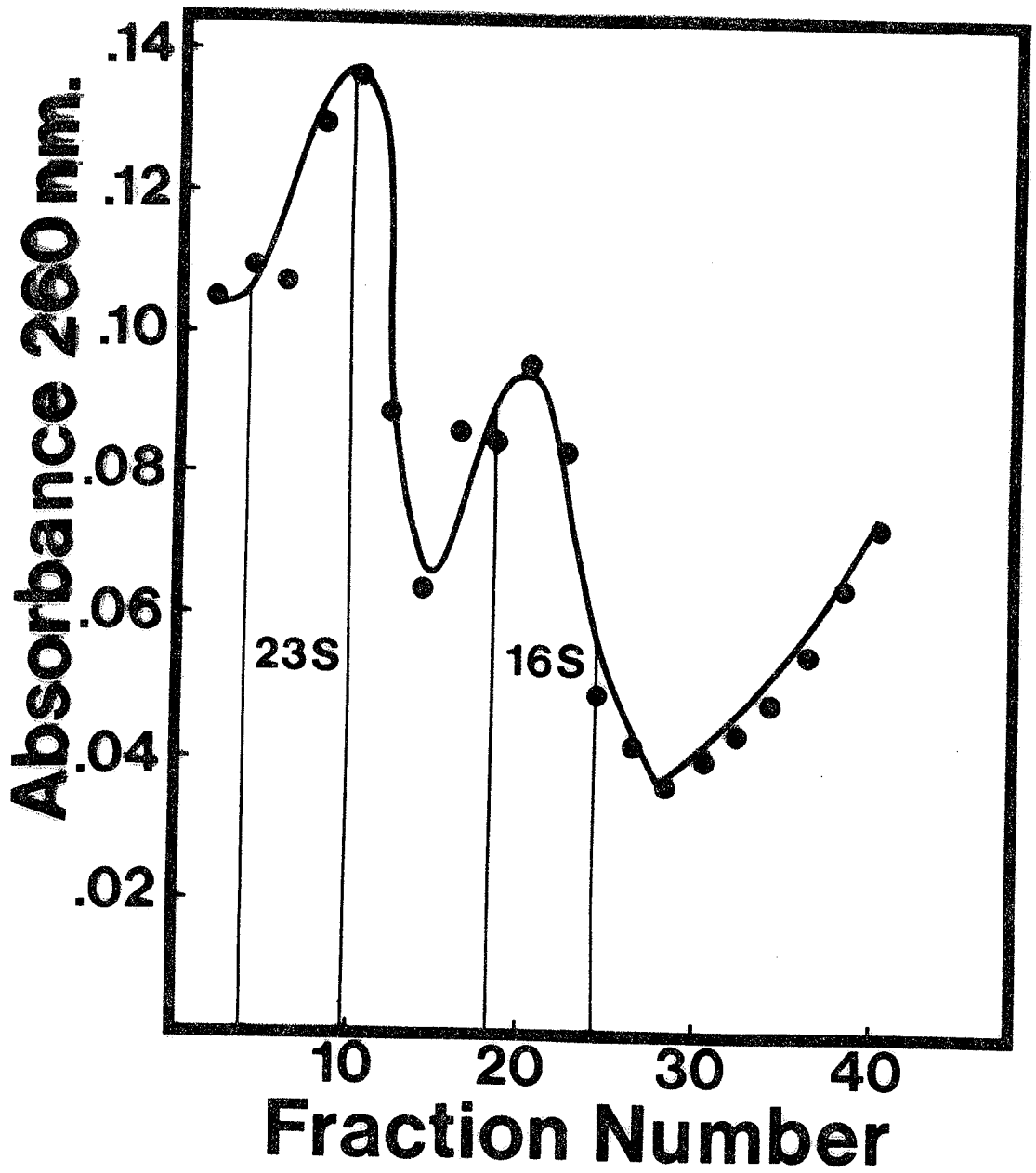
RNA Extraction and Purification

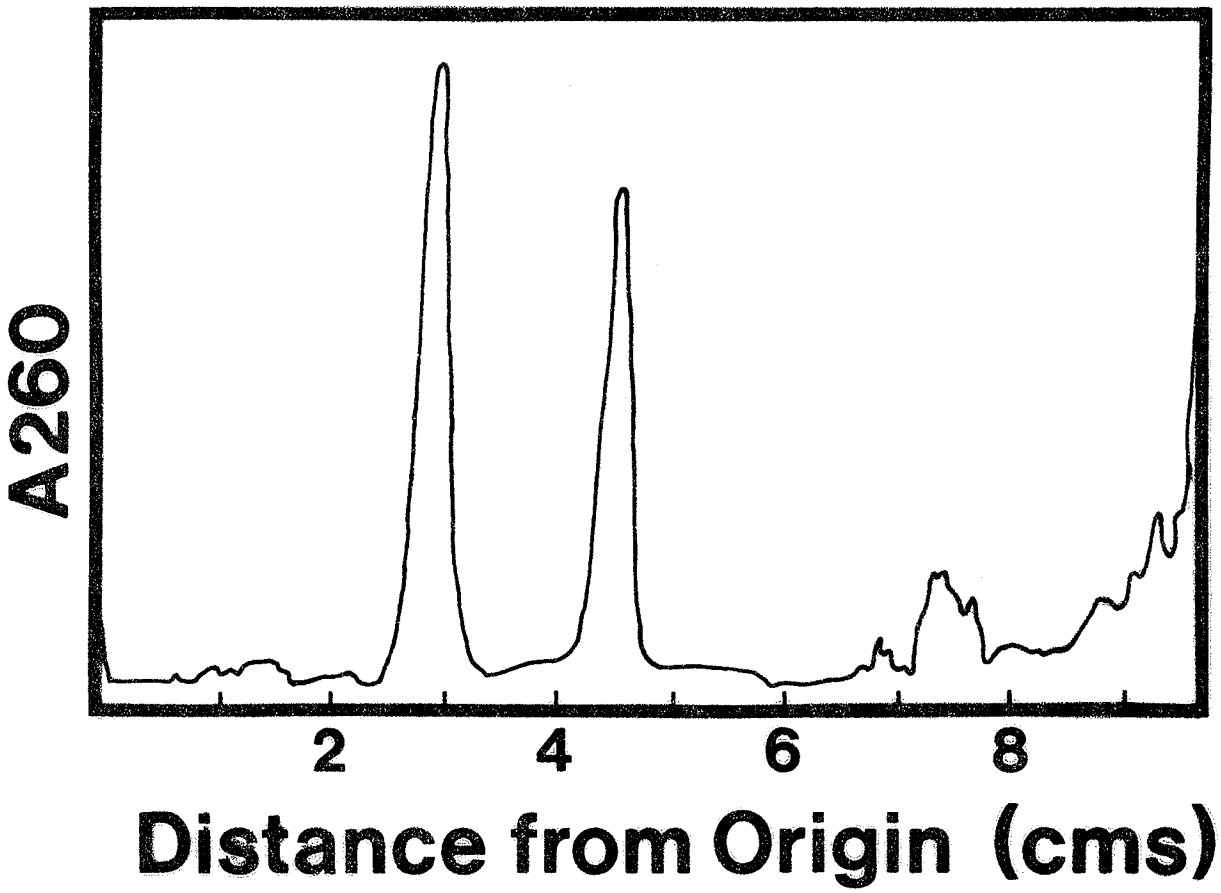
Figure 2 shows the growth curve for Myxococcus xanthus MD2 grown in CM medium at 32°C as described in Methods. The cells grew with an average generation time of 240 minutes to a maximum density of 350-400 Klett units (red filter). When used for RNA extraction, cultures were harvested during the late log phase of growth when their density reached approximately 250 Klett units. Generally, cells from 2.4 liters of medium (approx. 15 g of cells) were used for the extraction process.

Ribosomal RNA was extracted from the cells as described in Methods. To minimize degradation of RNA by nucleases, SDS was incorporated, where possible, into reagents used for extraction. The extracted RNA was separated into 16 and 23S RNA's by centrifugation through a 60 ml linear sucrose gradient as described in Methods. Figure 3 shows the sedimentation profile of a typical run as well as the fractions that were pooled. The A_{260}-absorbing material at the top of the gradient probably represents messenger and transfer RNA's and DNA fragments that were not extracted with 3.0 M Na acetate. The amount of this material varied with each extraction. If the pooled 16S RNA was contaminated, it was subjected to a second sucrose gradient centrifugation.

The purity of the RNA was checked by polyacrylamide gel electrophoresis (Methods). Figure 4 shows the electrophoretic profile of ribosomal RNA before sucrose gradient centrifugation. As can be seen from the Figure, this particular batch of RNA was not as heavily contaminated as that shown in Figure 3. The jagged peak evident between 7 and 8 cm


500
400
300
200
100
50
10
Klett Units
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)


Absorbance 260 nm.
.14
.12
.10
.08
.06
.04
.02
23S
16S
10
20
30
40
Fraction Number

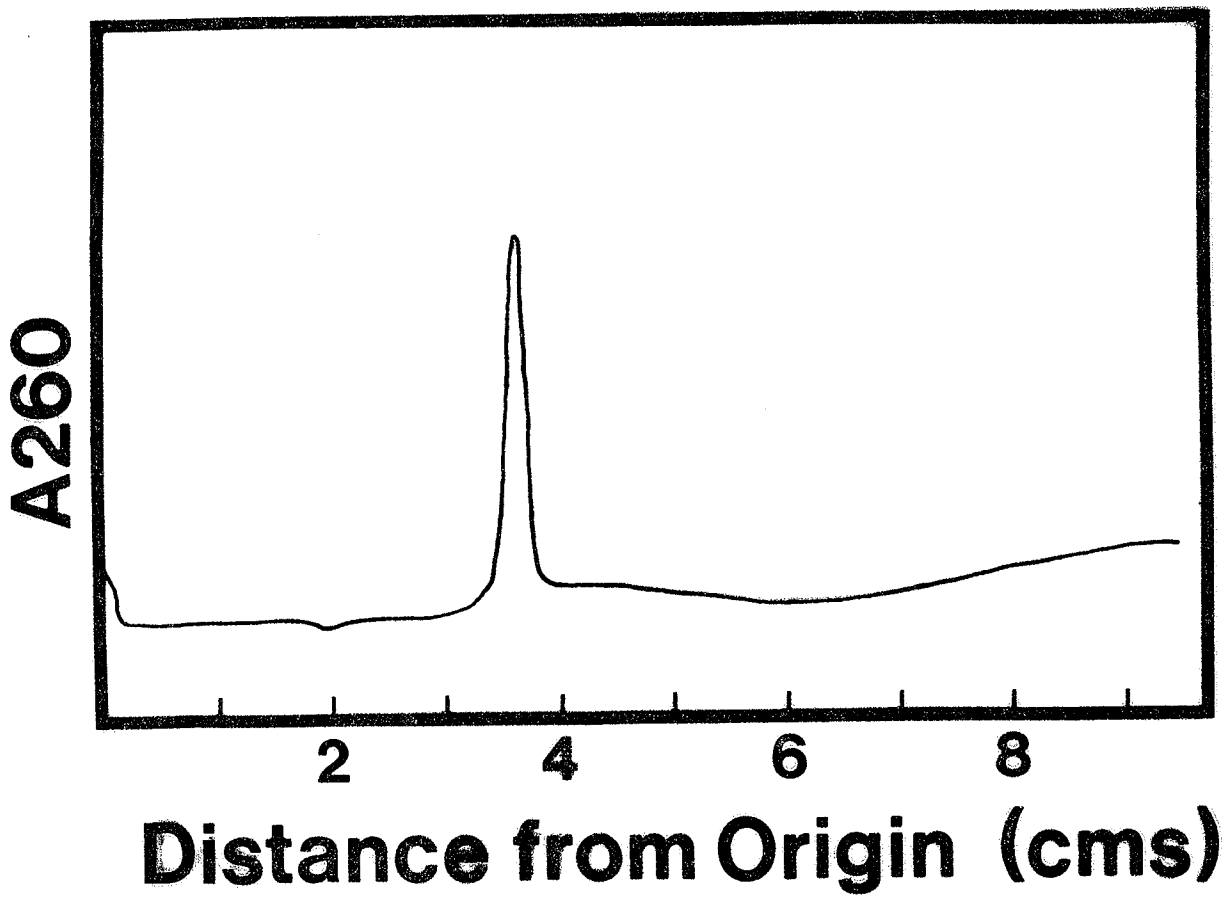

A260
2
4
6
8
Distance from Origin (cms)

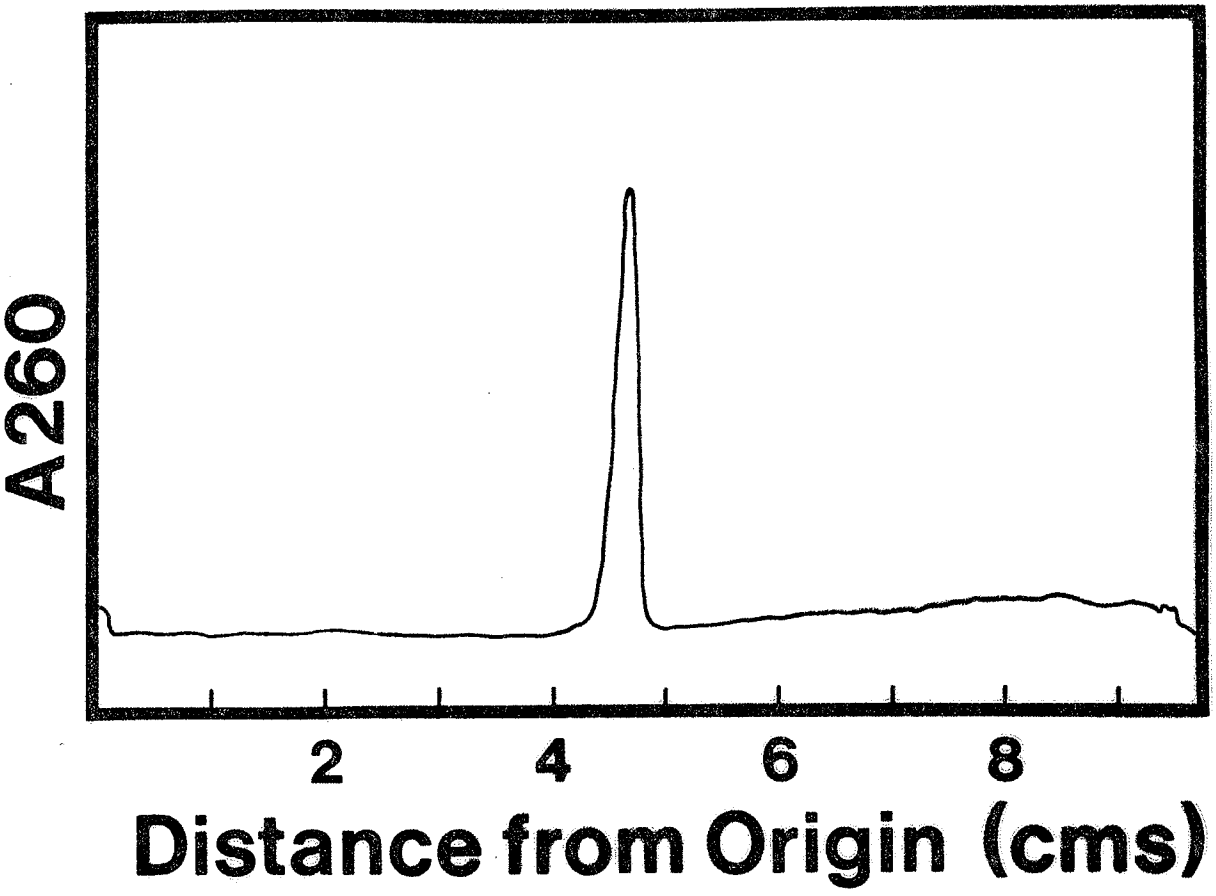
from origin (Fig. 4) is due to a break in the gel.

After 1 or 2 cycles of sucrose gradient centrifugation, the 16S RNA was essentially free of 23S RNA. This is shown by the electrophoretic profile of purified 16S RNA (Fig. 5). It was necessary to determine the integrity of the RNA since any breaks resulting in exposed 3'-OH ends would be labelled in subsequent steps. Such breaks would not be revealed by normal polyacrylamide gel electrophoresis, because the fragments of 16S RNA may be held together by hydrogen bonds and the RNA would migrate as a single band. Consequently, purified 16S RNA was heated for 1 min at 80°C to 0.1% SDS to disrupt hydrogen bonding then cooled rapidly in ice. The heat-denatured sample migrated as a single 16S peak (Fig. 6) and was judged to be intact.

Purified 16S RNA was also isolated from cells that had been induced to sporulate with glycerol for 60 and 120 min (Methods). After 60 min in glycerol, the cells had become shorter and by 120 min virtually all cells (95%) appeared as spherical myxospores. The effect of glycerol on vegetative cells has been reported by Dworkin (1972). The isolation of 16S RNA from sporulating cells was the same as described for vegetative cells except they were disrupted by grinding with alumina (Methods). This was required since sporulating cells are resistant to lysis by SDS. The purity and integrity of 16S RNA from these cells was the same as that from vegetative cells (not shown).

The 16S RNA from vegetative cells was oxidized as described in Methods with a 100-fold molar excess of sodium metaperiodate to insure complete oxidation. The RNA was precipitated with alcohol to remove



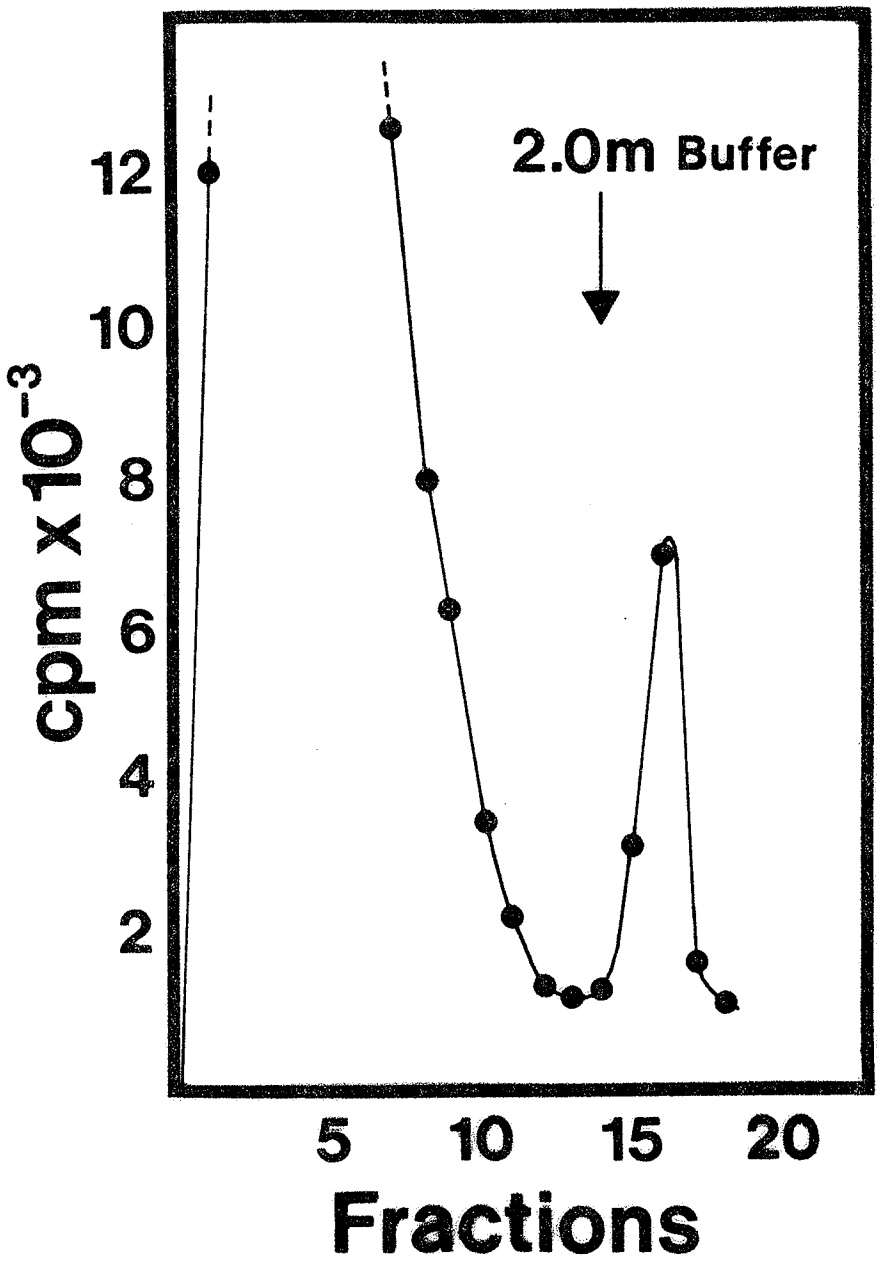

A260
2
4
6
8
Distance from Origin (cms)

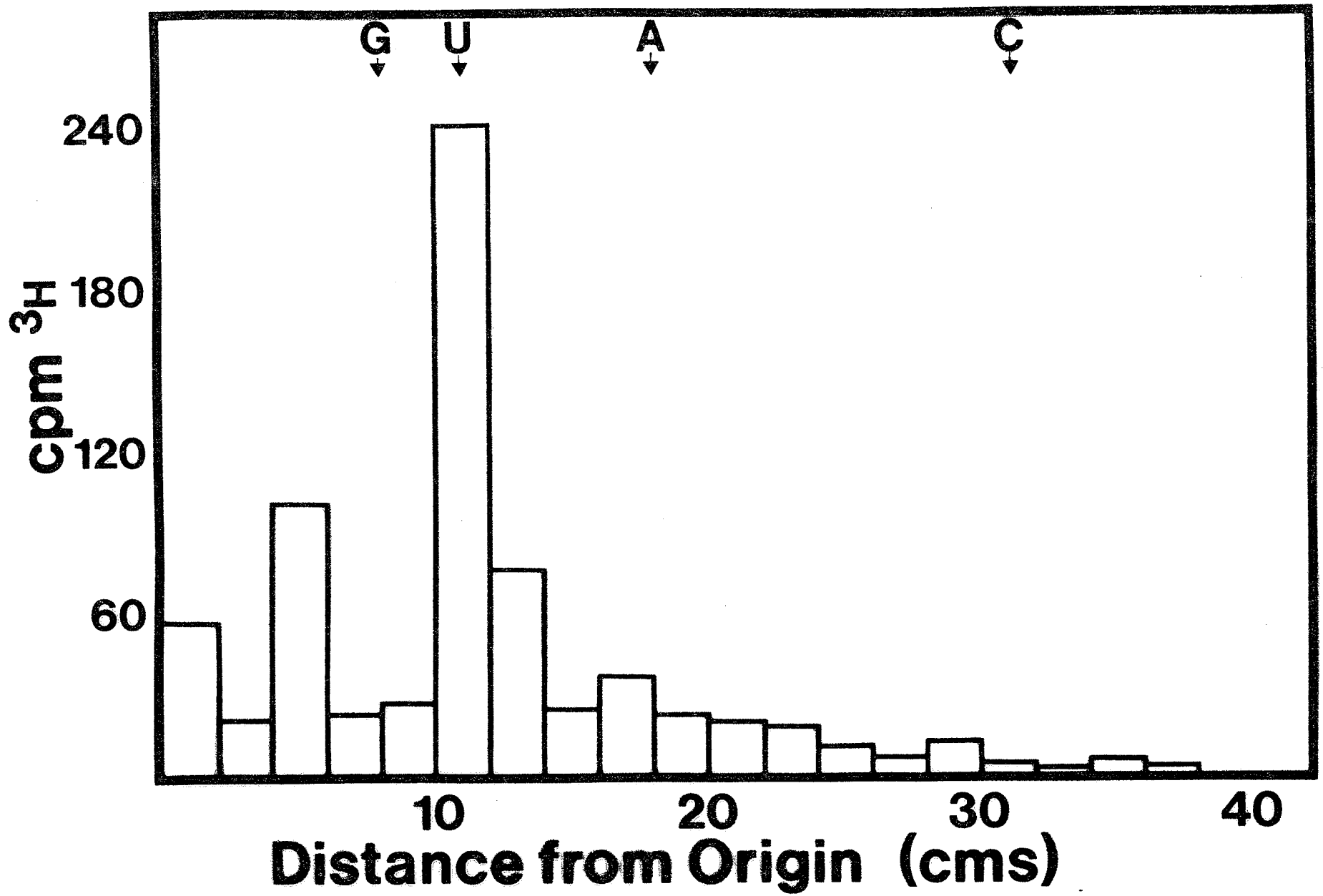
unreacted periodate then dissolved in buffer suitable for condensation with ^{3}H-isoniazid or for degradation with aniline.

The oxidized 3'-hydroxyl terminus was labelled by condensation with ^{3}H-isoniazid (Methods). Excess isoniazid was removed by chromatography on DEAE-cellulose (Methods). Figure 7 illustrates the separation of isoniazid which was washed from the column by 0.1 M Na acetate (pH 5.0) and the labelled RNA that was eluted with 2.0 M Na acetate (pH 5.0). The RNA was precipitated from the pooled fractions and dissolved in Na/K phosphate buffer (0.01 M, pH 7.4) at a concentration of 1 mg/ml.

Digestion of labelled RNA was then carried out with a mixture of ribonucleases T1 and A as described in Methods. The combination of enzymes will generally yield mononucleosides. However, some oligonucleotides containing adenosine will also be generated since the enzymes are unable to hydrolyze 3'-phosphoester bonds of adenosine residues. However, these can be distinguished by electrophoresis (Shine and Dalgarno, 1974). To identify the ^{3}H-isoniazid-labelled nucleoside, the digest was mixed with standard nucleoside isonicotinoyl hydrazones (Methods) and separated by paper electrophoresis (Methods). After electrophoresis, the positions of each of the nucleoside standards was located under ultraviolet light, then the electropherogram was cut into strips for scintillation counting (Methods). Figure 8 shows the profile of the electropherogram. The arrows in the Figure indicate the position of migration of the standard nucleoside isonicotinoyl hydrazones.

The radioactivity at the front of the electropherogram

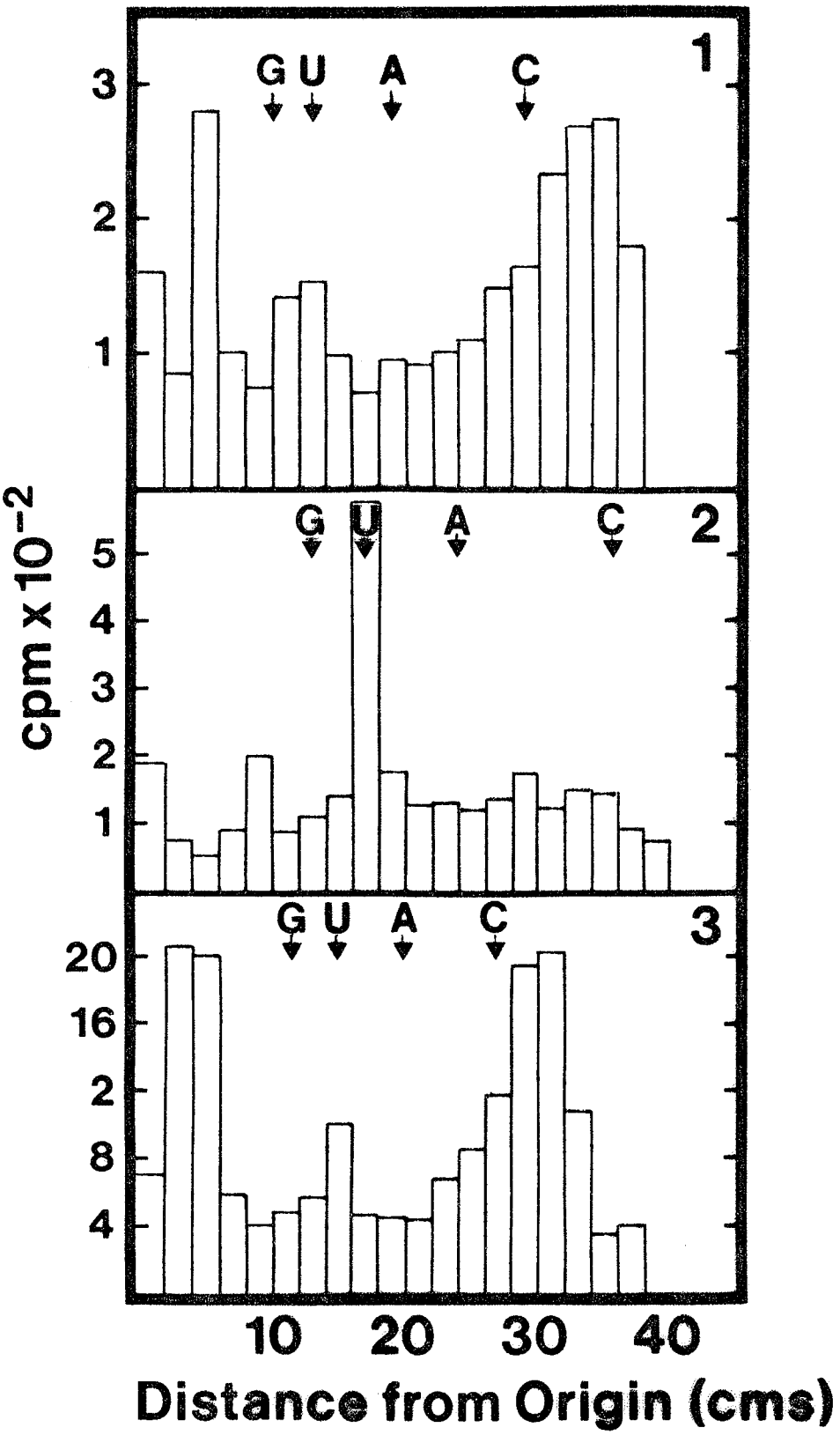

2.0m Buffer
12
10
8
6
4
2
cpm x10⁻³
5
10
15
20
Fractions

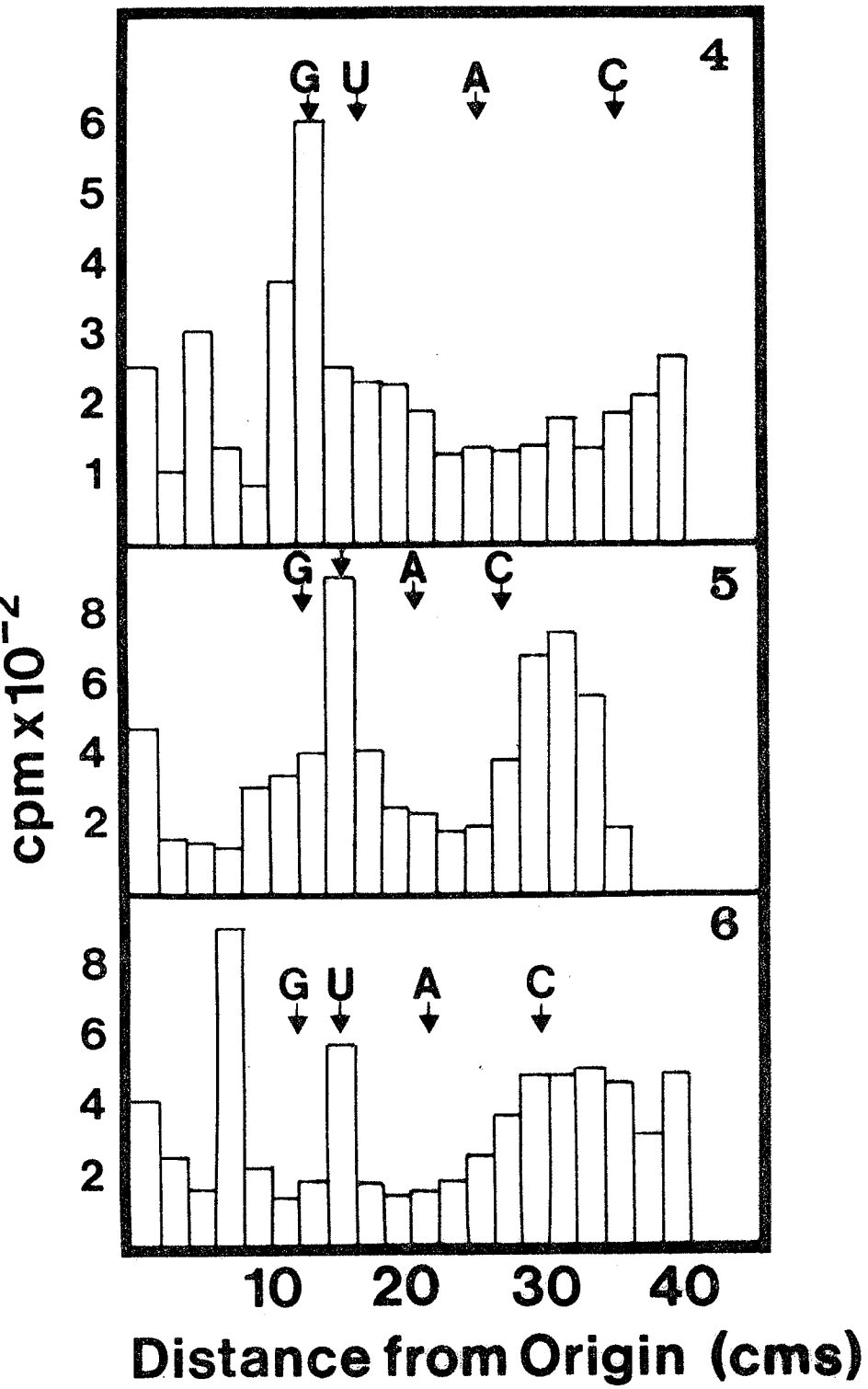

G
U
A
C
240
180
120
60
cpm ^{3}H
10
20
30
40
Distance from Origin (cms)

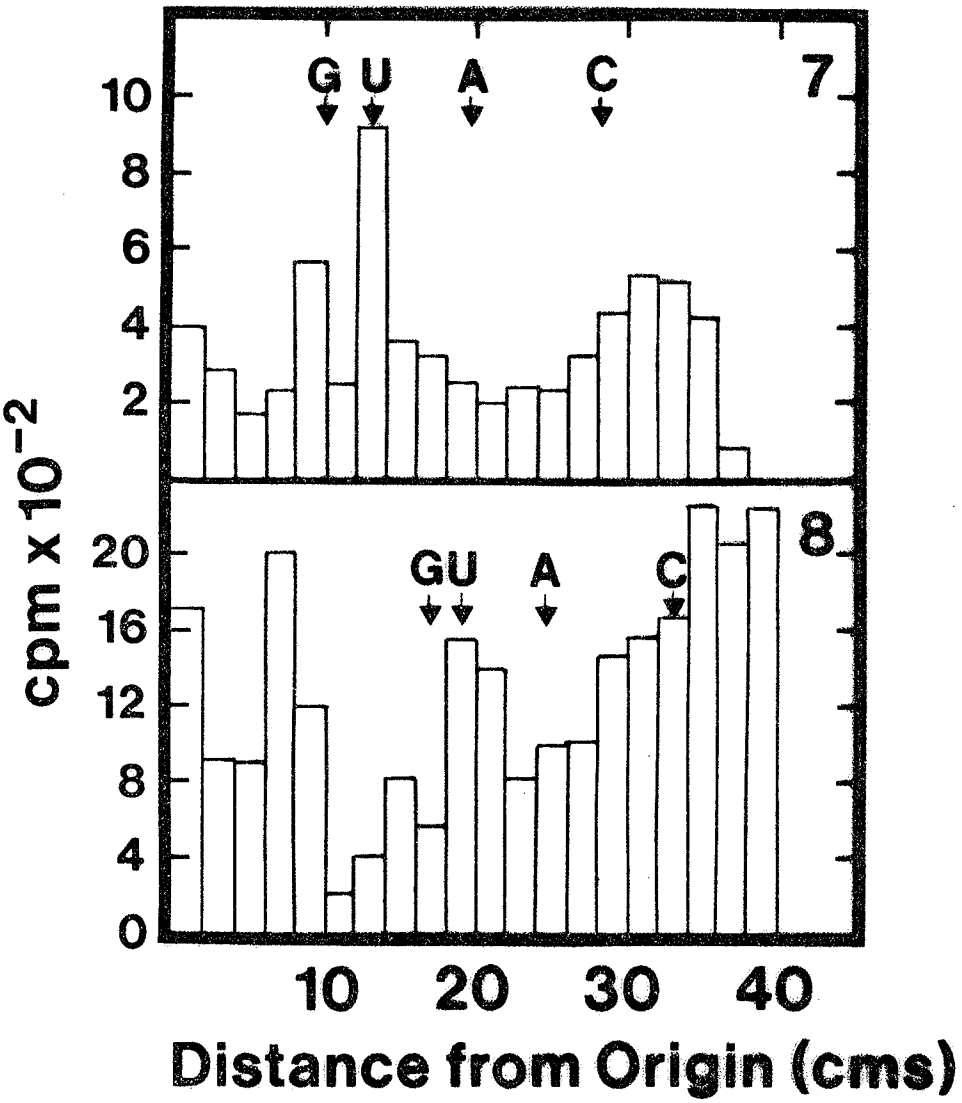
represents free ^{3}H-isoniazid and that within 5 cm of the origin is a contaminant present in isoniazid. These were identified by electrophoresis of ^{3}H-isoniazid, alone, under identical conditions. Shine and Dalgarno (1974) have also reported the appearance of the isoniazied peaks. Some radioactivity co-electrophoresed with the non-radioactive marker uridine isonicotinoyl hydrazone. This establishes the 3'-terminal nucleotide in vegetative 165 RNA as uridine.

To determine the subterminal nucleotide, the periodate-oxidized 16S RNA (above) was treated with aniline and alkaline phosphatase (Methods) to remove the terminal nucleotide and create a new 3'-OH terminus. This 16S RNA (n-1, where n= number of nucleotides in the original RNA) was oxidized with periodate (Methods). A portion was condensed with ^{3}H-isoniazid, digested with ribonucleases (T1 and pancreatic); then the digest was analyzed by paper electrophoresis as above. The remainder of the ozidized 16S RNA (n-1) was treated with aniline and alkaline phosphatase to generate n-2 16S RNA. By repetitive cycles of treatment it is possible to remove one nucleotide at a time from RNA, label each new terminus with ^{3}H-isoniazid and identify the terminal nucleotide by paper electrophoresis of ribonuclease digests of the labelled RNA.

The electropherograms of ribonuclease digests of ^{3}H-isoniazid-labelled n-1 to n-8 16S RNA of vegetative cells is shown in Figures 9, 10 and 11. In these figures, the number in the top left corner of each electrophoretic profile designates the number of aniline treatments the RNA had been subjected to. Surprisingly with the exception of the n-4 RNA (Fig. 10.4), all terminal nucleotides were identified as uridine.


G U A C
1
3
2
1
G U A C
2
5
4
3
2
1
G U A C
3
20
16
2
8
4
cpm x 10⁻²
10
20
30
40
Distance from Origin (cms)

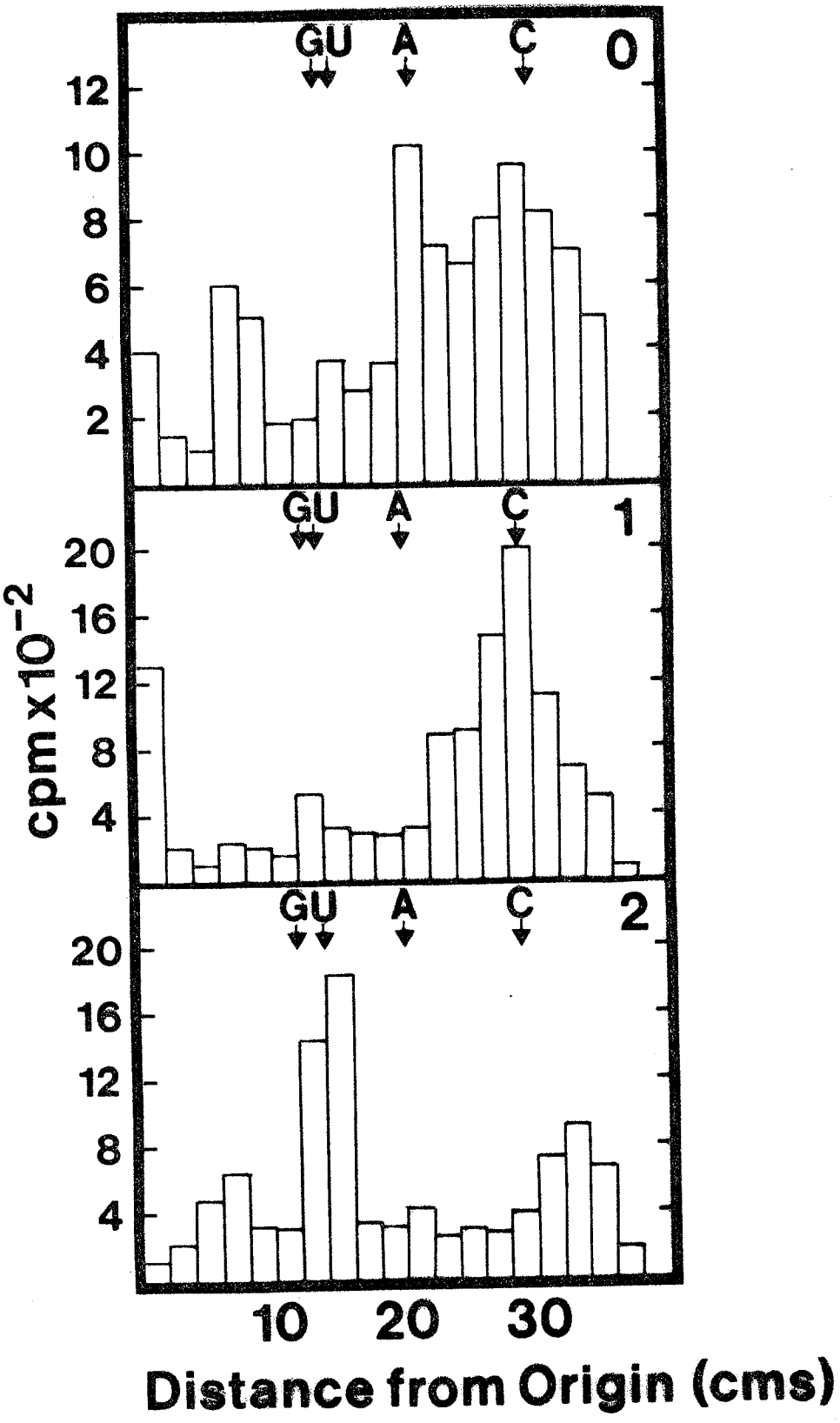

4
G U A C
6
5
4
3
2
1
5
G A C
8
6
4
2
6
G U A C
8
6
4
2
cpm x10^{-2}
10 20 30 40
Distance from Origin (cms)


G U A C
7
10
8
6
4
2
GU A C
8
20
16
12
8
4
0
cpm x 10⁻²
10 20 30 40
Distance from Origin (cms)

The sequence of the 16S RNA is likely $_{HO}$U-U-U-U-G-U-U-U-U.

As can be seen from the electropherograms, the isonicotinoyl hydrazones of guanine and uracil migrate very close together and are difficult to distinguish. However, each digest is co-electrophoresed with each of the standard nucleoside isonicotinoyl hydrazone and in each of the 4 profiles obtained, the radioactive nucleoside hydrazone runs more with uracil than guanine. It is, in fact, more difficult to distinguish cytidine isonicotinoyl hydrazone since the free isoniazid tails and may mask this region. For instance, in Figures 9.1, 9.3, 10.6, and 11.8, where the "U" peak is depressed in comparison with "C" and free isoniazid peak, it may be that the terminal nucleotides may be cytidine rather than uridine. Efforts to improve electrophoretic resolution by altering buffer molarity and pH and running conditions (voltage and time) were uniformly unsuccessful.

To test the validity of the method the 3'-termini of E. coli transfer RNA was sequenced using the same method. The method should yield a $_{HO}$A-C-C sequence. The electropherograms of ribonuclease digests of ^{3}H-isoniazid-labelled tRNA before aniline treatment and after one and two treatments with aniline. The first nucleotide was adenosine (Fig. 12.0) and the second, cytidine (Fig. 12.1). The third nucleoside should also be cytidine, but, in fact, the bulk of the radioactivity migrates slightly ahead of uridine. In all probability, this represents a di-nucleoside $_{HO}$C-A in which the cytidyl residue is labelled with isoniazid (Shine and Dalgarno, 1974). This result is consistent with the inability of the mixture of ribonucleases employed to cleave at the 3'-side of


GU
A
C
0
12
10
8
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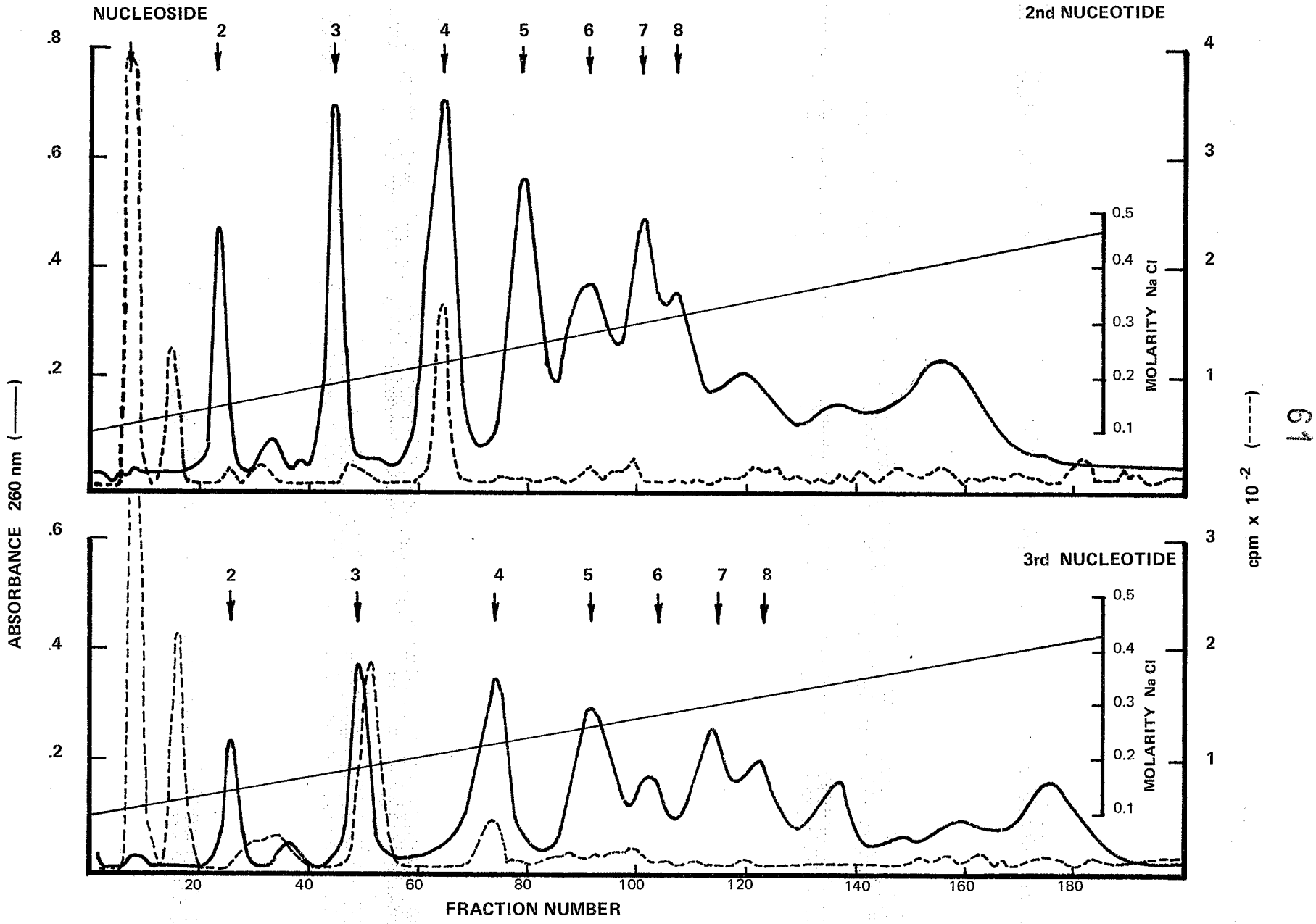
1
20
16
12
8
4
2
cpm x 10⁻²
10
20
30
Distance from Origin (cms)


NUCLEOSIDE
2nd NUCEOTIDE
3rd NUCLEOTIDE
2
3
4
5
6
7
8
ABSORBANCE 260 nm (——)
cpm x 10⁻² (- - - -)
MOLARITY Na Cl
0.1
0.2
0.3
0.4
0.5
.2
.4
.6
.8
1
2
3
4
20
40
60
80
100
120
140
160
180
FRACTION NUMBER

adenosine and indicates that in a large proportion of tRNA's, the 3'-sequence is $_{HO}$A-C-C-A.

The effectiveness of aniline in removing 3'-terminally oxidized nucleosides was tested. After each cycle of labelling, the RNA was digested with ribonuclease T1, alone, and the resulting fragments were separated on A-25 DEAE Sephadex as outlined in Methods. Since this procedure separated the fragments according to size (Tener, 1967), with each cycle of degradation, labelling and ribonuclease T1 digestion, the fragment bearing the isonicotinic-acid hydrazone should decrease in size by 1 nucleotide. Figure 13 shows the elution profile of labelled 16S RNA after the first (top, Fig. 13) and second (bottom, Fig. 13) aniline treatment in comparison with ribonuclease T1-digested carrier RNA. The elution positions and the number of nucleosides in the oligonucleotides are indicated by the arrows. The elution positions of nucleosides, dinucleoside monophosphates and trinucleoside diphosphates were determined as described in Methods and served as references for determining the size of other oligonucleotides (Hunt, 1973). The elution profile for only the first 200 (of 240-250) fractions is shown. Beyond this, no radioactivity or 260 nm-absorbing material was eluted, even when the column was washed with 2.0 M NaCl.

After labelling the second nucleotide, ribonuclease T1 digestion yielded a tritiated fragment that co-chromatographed with tetranucleoside triphosphates (top, Fig. 13). After a second cycle, in which the 3rd nucleotide was labelled, ribonuclease T1 digests yielded a labelled fragment corresponding to a trinucleoside diphosphate (bottom,

Fig.13). This clearly shows aniline treatment removed one nucleoside from the 3'-end. The minor peak appearing in fraction 70-75 (bottom, Figure 13) indicates the removal was not complete. Successive cycles of treatment further reduced the size of the fragment in a stepwise fashion (not shown). The results indicate that the 5th nucleotide from the 3'-end of 16S RNA is likely a guanosine.

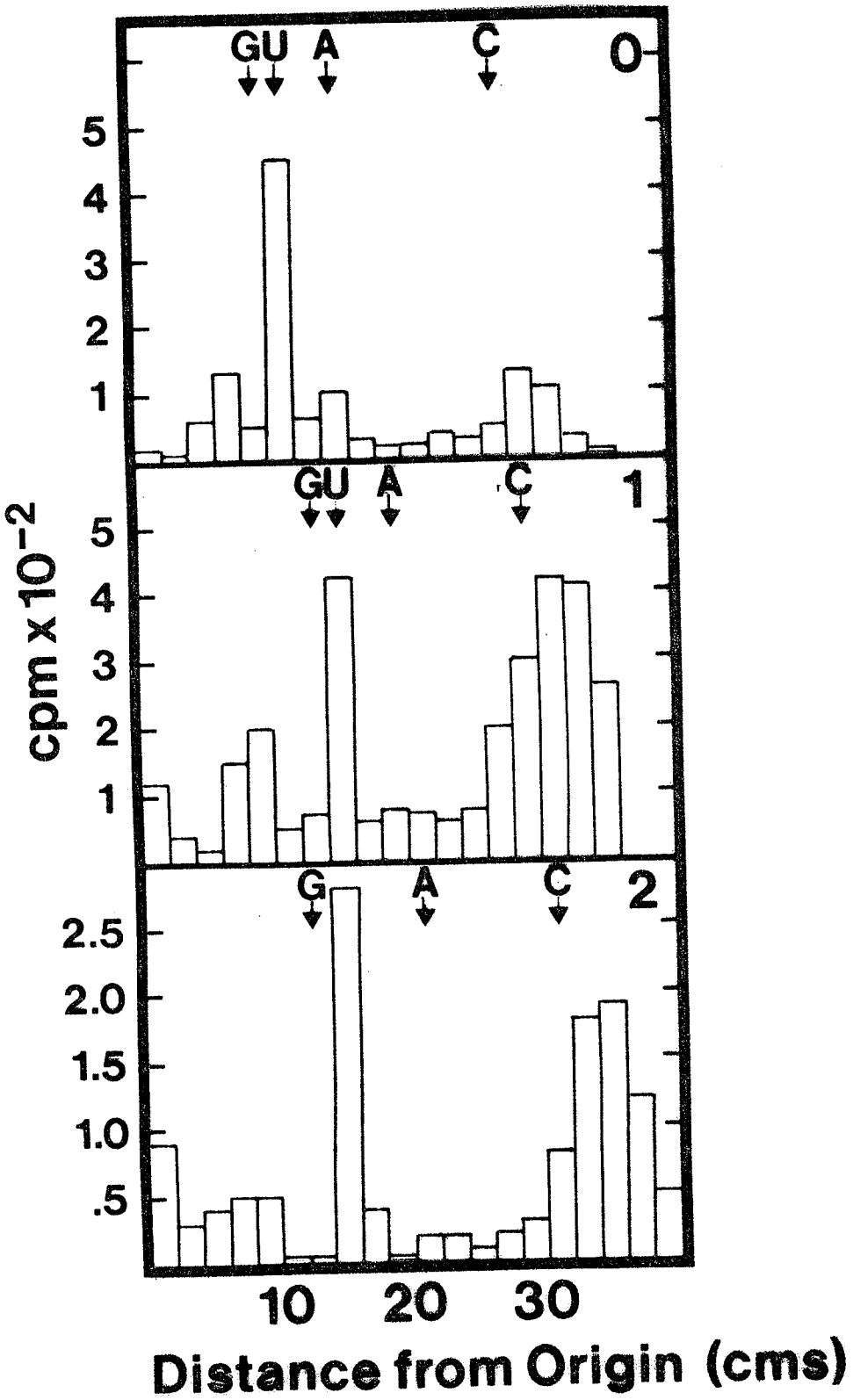
The radioactivity peak eluting at the void volume (fractions 7-9) represented unreacted ^{3}H-isoniazid (Hunt, 1973) and co-eluted with adenosine. The radioactivity peak between the nucleoside and dinucleoside monophosphate probably represented a contaminant in the ^{3}H-isonicotinic acid hydrazide stock, since it appeared all eight of the chromatographic determinations made, regardless of the stage at which labelling was carried out. It was not a nucleoside monophosphate, which at this pH would co-migrate with trinucleoside diphosphates. The results show that aniline treatment cleaves single nucleosides from the 3'-end of RNA and confirms the presence of a guanosine residue as the fifth nucleotide from the 3'-end.

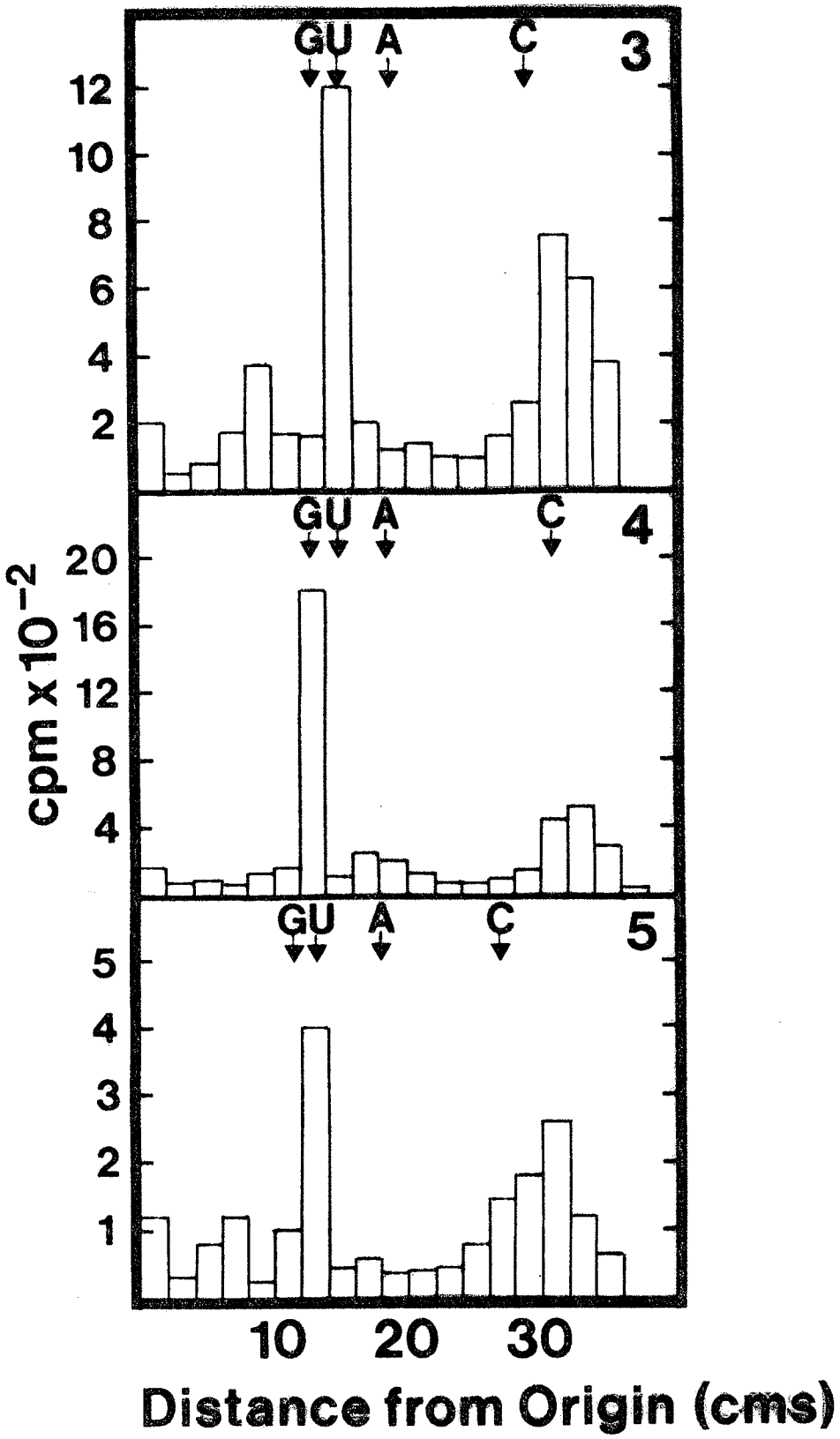
To determine whether a species of 16S RNA with a different sequence at the 3'-end is formed during sporulation, the sequencing procedure was repeated with 16S RNA isolated from cells induced to sporulate for 60 and 120 minutes. Induction was carried out by addition of glycerol to actively growing vegetative cells as described in Methods. Sixty minutes after induction, most of the cells (80-90%) were noticeably shorter, and by 120 minutes, 90% of the cells appeared as spherical myxospores with average diameters of around 5μm. At the latter stage

most RNA metabolism ceases (Bacon and Rosenberg, 1967). Ribosomal 16S RNA was isolated from these cultures as described in Methods.

The RNA was subject to stepwise degradation as described above, and at each step, the 3'-end was labelled with ^{3}H-isoniazid. The labelled RNA was digested with ribonucleases and the ^{3}H-nucleoside isonicotinoyl hydrazone was identified by high voltage paper electrophoresis. Figures 14, 15 and 16 show the electrophoretic profile of ribonuclease digests of 16S RNA isolated from cells 60 min after addition of glycerol. The number in the top right of each figure designates the number of aniline-catalyzed degradations to which the RNA had been subjected. The sequences for the RNA is, therefore, U-U-U-G-U-U-U-U_{OH} and is identical to that of 16S RNA from vegetative cells.

In the final set of experiments the sequence of nucleotides was deduce for the 3'-end of 16S RNA isolated from cells 120 min after induction. At this stage myxospores are indistinguishable from those found in fruiting bodies (Dworkin, 1965), and synthesis of ribosomal RNA has ceased (Bacon and Rosenberg, 1967). The isolation of the RNA is outlined in Methods. The RNA was degraded with aniline and labelled with ^{3}H-isoniazid as described previously. The labelled RNA was digested with ribonucleases and nucleoside isonicotinoyl hydrazones were identified by paper electrophoresis. Figure 17, 18 and 19 show the electrophoretic profiles obtained from these digests. The number in the top right indicates the number of aniline treatments to which the RNA had been subjected and, therefore, the number of nucleotides cleared from the 3'-end before labelling with ^{3}H-isoniazid. From the figures the sequence


GU A C O
5
4
3
2
1
GU A C 1
5
4
3
2
1
G A C 2
2.5
2.0
1.5
1.0
.5
cpm x 10⁻²
10 20 30
Distance from Origin (cms)


GU
A
C
3
12
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2
GU
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4
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16
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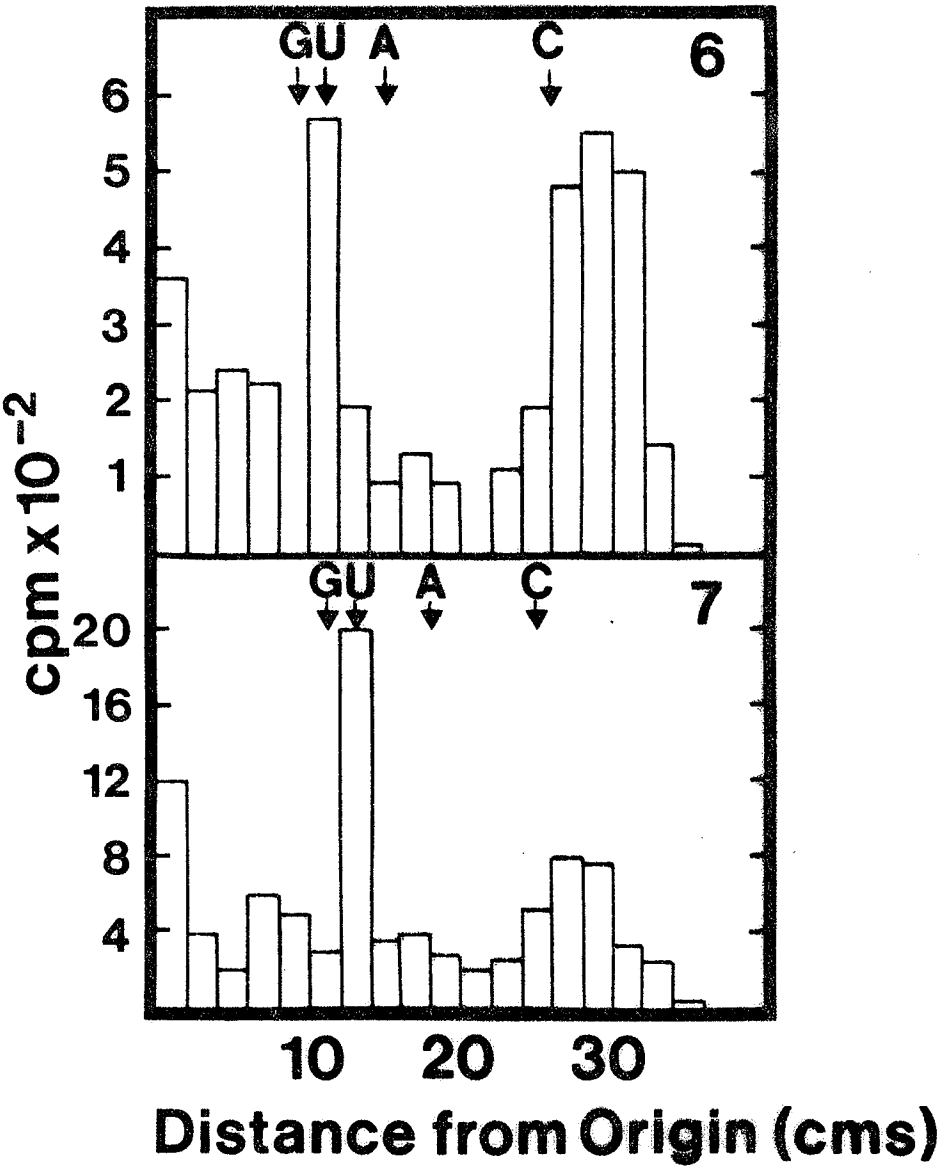
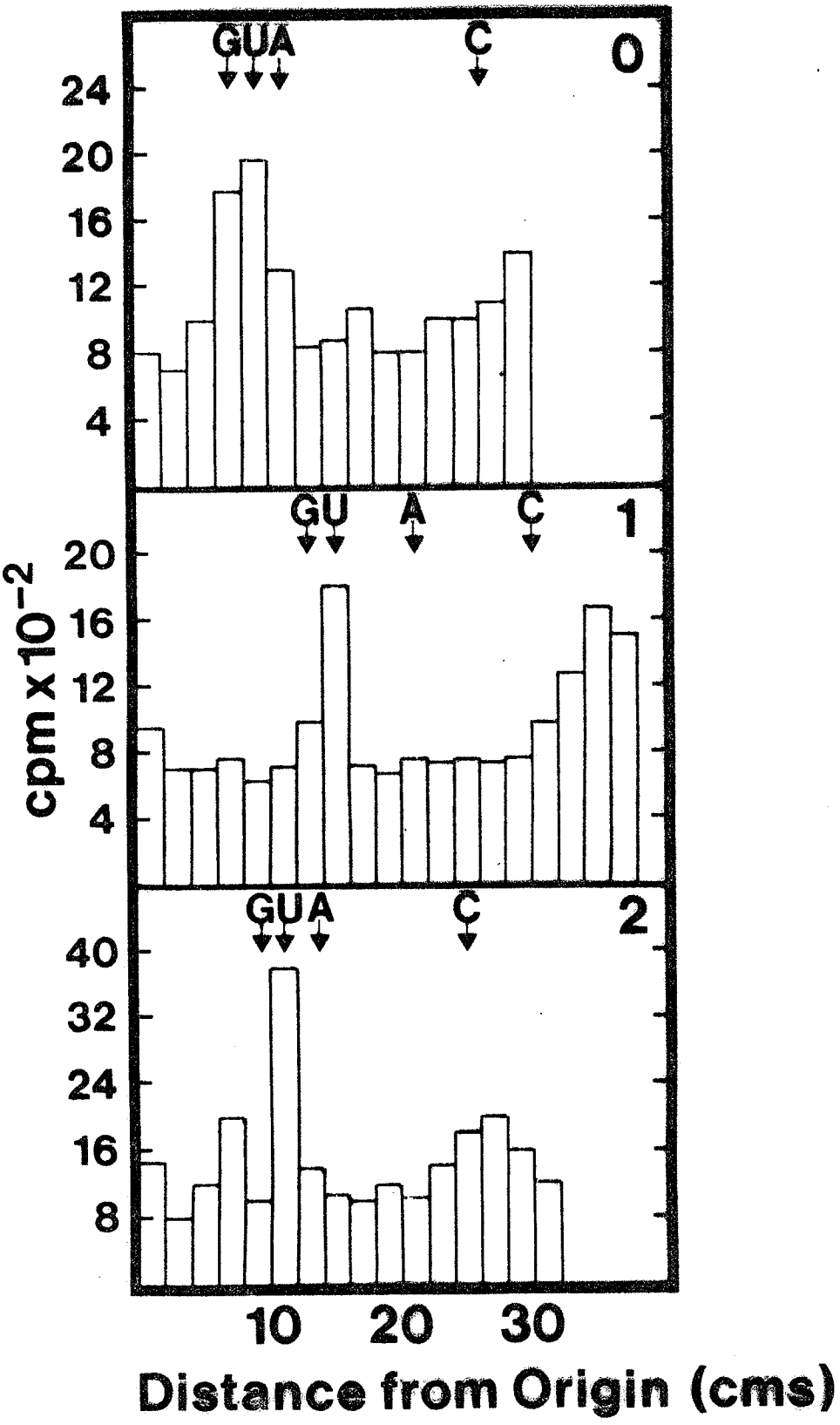
GU
A
C
5
5
4
3
2
1
cpm x 10⁻²
10
20
30
Distance from Origin (cms)


GU
A
C
6
7
cpm x10^{-2}
Distance from Origin (cms)


GUA
C
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20
16
12
8
4
GU
A
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1
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16
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8
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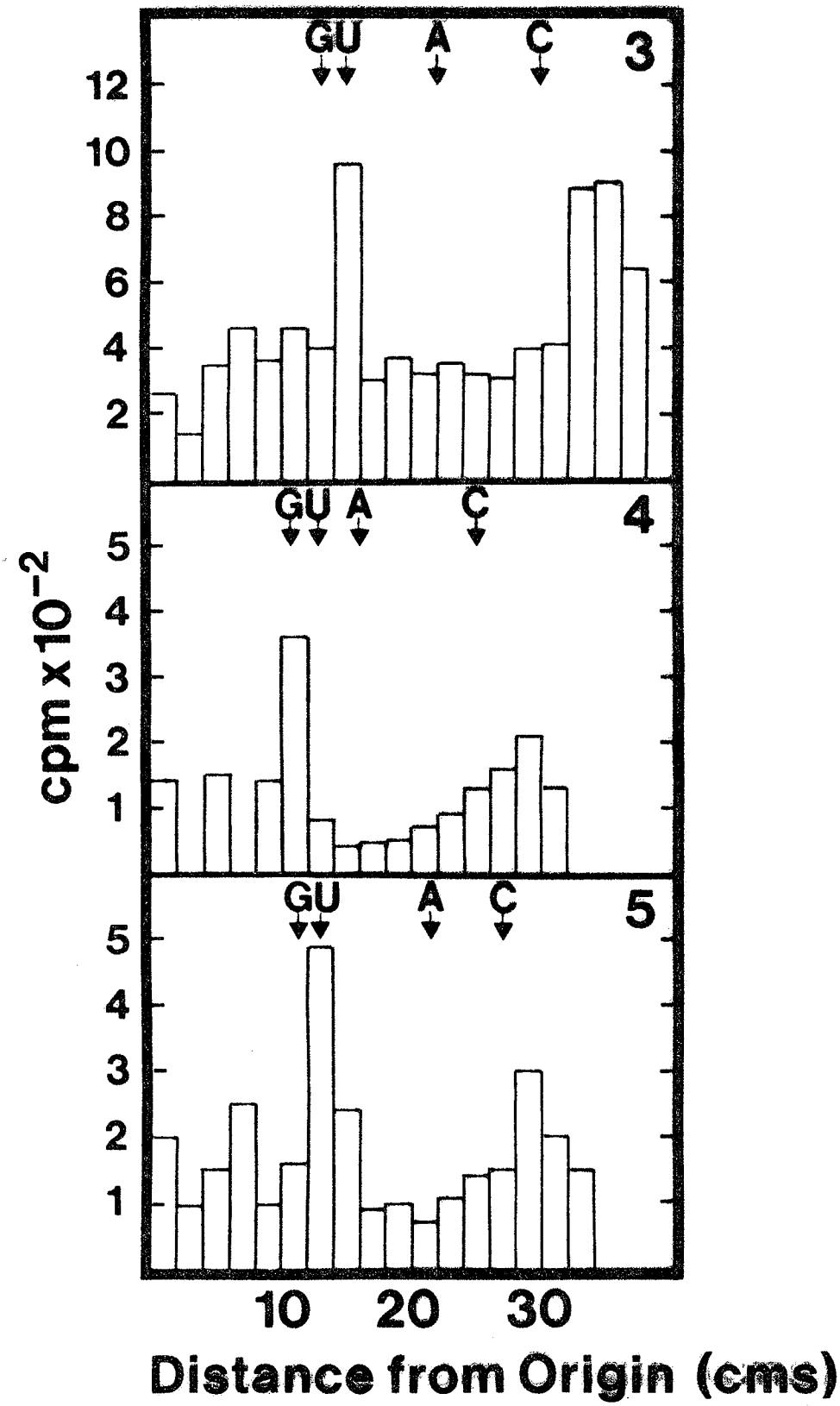
GUA
C
2
40
32
24
16
8
cpm x10^{-2}
10
20
30
Distance from Origin (cms)


GU
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C
3
12
10
8
6
4
2
GU
A
C
4
5
4
3
2
1
GU
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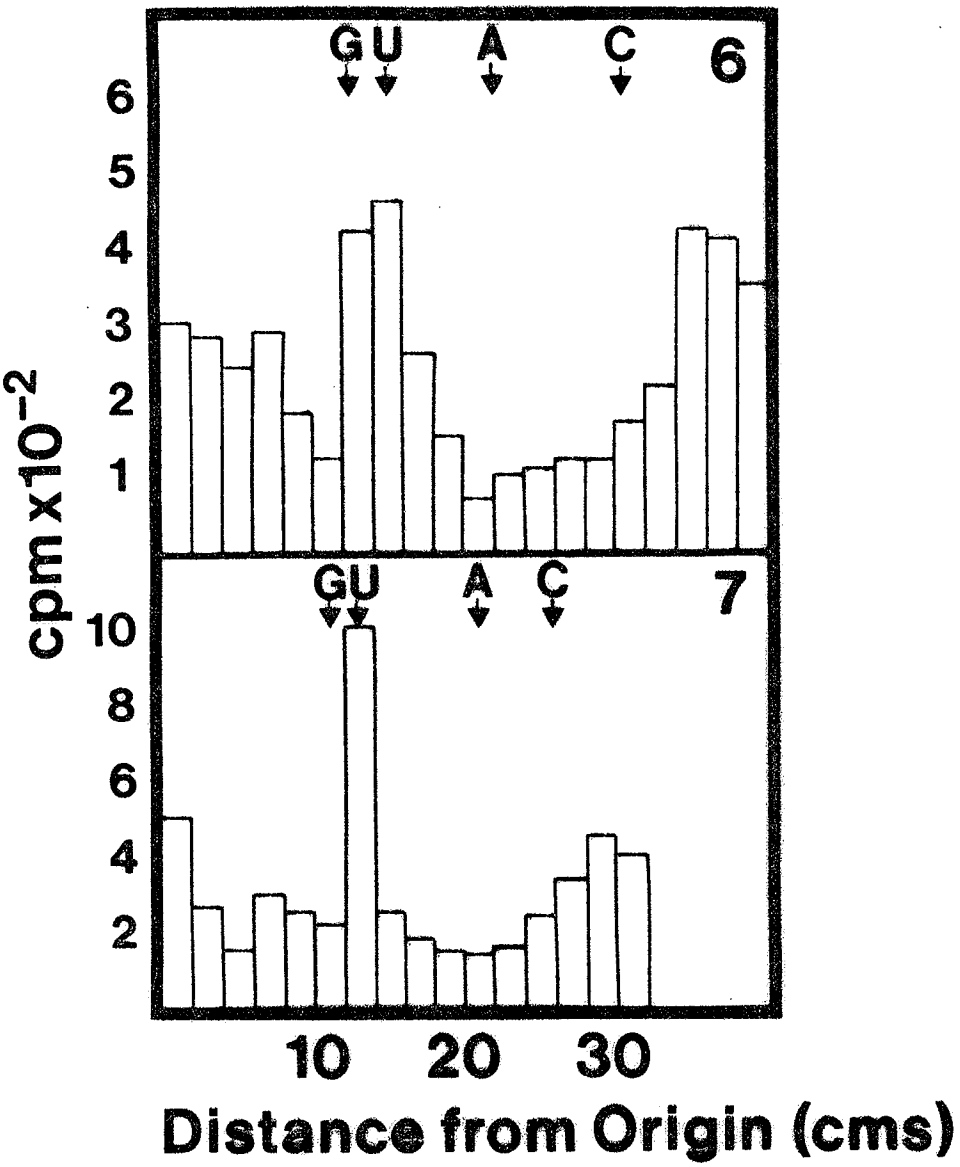
C
5
5
4
3
2
1
cpm x10⁻²
10
20
30
Distance from Origin (cms)


G U
A
C
6
6
5
4
3
2
1
cpm x10^{-2}
G U
A
C
7
10
8
6
4
2
10
20
30
Distance from Origin (cms)

$_{HO}$U-U-U-U-G-U-U-U was deduced. It is identical to the 3'-sequence in 16S RNA's isolated from vegetative cells and cells induced to sporulate for 60 min.

DISCUSSION

The study of the 3'-end of 16S ribosomal RNA during development of Myxococcus xanthus did not disclose any changes in the nucleotide sequence $_{HO}$U-U-U-U-G-U-U-U-U-(5'), determined for vegetative cells. Since the accuracy of this sequence depended on the condition of isolated 16S RNA, care was taken to ensure that the purified 16S RNA was free of scissions and contaminating material such as fragments of 23S RNA, DNA and polysaccharide. Contamination of RNA with polysaccharide was a possibility in view of the observation that the cells secrete copious quantities of slime in the course of growth (Wireman and Dworkin, 1975). Ceri and Maeba (1973), have also reported that the 23S rRNA of E. coli was rapidly degraded by a ribonuclease associated with the 50S ribosomal subunit. An analogous process in M. xanthus may have contributed fragments of 23S RNA.

In view of the possibility of contamination of 16S RNA, the extraction procedure was modified to ensure the isolation of pure 16S ribosomal RNA. Most of the contaminating polysaccharide and DNA was removed with cold, 3.0 M Na acetate, which also contained 0.1% SDS (w/v). Sodium-dodecyl sulfate was also found useful during high speed centrifugation of RNA in sucrose gradients, a process which removed residual polysaccharide and DNA (see Methods).

The purity and integrity of 16S rRNA was evaluated with SDS-acrylamide gel electrophoresis. Following each 16S RNA isolation, a portion of the RNA was electrophoresed. The appearance of a single peak with the migration profile of 16S rRNA, and the absence of other absorbing

material attested to the purity of the isolated material. Moreover, electrophoresis of heat-denatured 16S rRNA on SDS-acrylamide gels confirmed the lack of scission in 16S RNA.

In view of the appearance of long tracts of poly-(U), it was necessary to demonstrate that aniline successfully removed the oxidized, terminal nucleoside. This was accomplished by sequencing the 3'-end of yeast transfer RNA as a control. In addition, the aniline reaction was evaluated by chromatography of terminally reduced RNA on DEAE-Sephadex A-25. The primary structure of E. coli tRNA was previously determined by RajBhandary et al., (1968), and the 3'-end was established to be $_{HO}$A-C-C-A(5'). When the 3'-end of transfer RNA was sequenced by the method of Hunt (1965), the results were found to be in full agreement with the sequence established by RajBhandary et al. The chromatographic analysis of subterminally-reduced RNA confirmed the removal of the terminal nucleoside by aniline catalysis (see Figure 13).

The finding of poly-(U) tracts at the 3'-end of 16S rRNA in M. xanthus was not unorthodox. The 3'-termini of several bacterial 16S rRNA molecules also contain long tracts of poly-(U) (see Table 2). Schroeder et al., (1977) have reported the presence of long poly-(U) tracts in the precursor of Bacillus subtilis 5S RNA. Moreover, in the course of transcription, six to seven adenylate residues at the termination site are copied into poly-(U) sequences at the 3'-end of mRNA (Doi, 1977). Bertrand et al., (1975) have reported the appearance of 8 uridylate residues at the 3'-end of mRNA of the E. coli trp leader. Thus, the poly-(U) rich segment at the 3'-end of 16S rRNA may reflect the

predominance of adenylate residues in the terminator regions of ribosomal genes.

The appearance of poly-(U) segments in the precursor of B. subtilis 5S RNA raised the possibility that the RNA sequenced was not mature 16S RNA, but a precursor. Indeed, the ribosomal genes in most bacteria exist in a tandem of 16S, 23S and sometimes also 5S rRNA (Perry, 1976). The 16S and 23S rRNA are transcribed together from the rDNA, with a large transcribed spacer between them (Perry, 1976). These precursor molecules are immediately tailored by ribonucleases, to generate mature molecules (Perry, 1976). Therefore, the possibility of isolating a precursor 16S rRNA was low, as they are rapidly tailored following synthesis (Perry, 1976). Moreover, while it has been demonstrated that E. coli precursor 16S rRNA migrated differently from mature rRNA, SDS-acrylamide electrophoresis of M. xanthus 16S rRNA demonstrated the presence of a single peak; no other material was observed.

Although Shine and Dalgarno (1975) offer a well-documented argument that it is the 3'-end of 16S rRNA that confers mRNA cistron specificity on ribosomes, the relationship of this selectivity to development of M. xanthus remains unsettled. While the primary structure of 16S rRNA was found to remain unchanged during development of M. xanthus, the possibility exists that the ability of sporulation-specific mRNA molecules to bind to the 3'-end of 16S rRNA is altered during devlopment of this organism. The sporulation-specific mRNA molecules could exhibit a greater degree of complementarity to the 3'-end of 16S RNA than their vegetative counterparts. This possibility is consistent with the Shine

and Dalgarno hypothesis (1975), which predicts a positive correlation between the amount of translation for a given mRNA, and the degree of complementarity between the 3'-end of 16S rRNA and the 5'-end of the mRNA.

A number of other possibilities exist for control of protein synthesis during development of M. xanthus. Alton and Lodish (1977), have proposed that a reduction in one of the components of protein synthesizing machinery, such as an initiation factor, could lead to cessation of translation of mRNA with a low affinity for ribosomes, and a preferrential inhibition of translation of other mRNAs.

Doi (1977), has described four additional mechanisms by which cells may bring about selective gene transcription. These include: synthesis of new RNA polymerases capable of recognizing sporulation-specific promoters; modification of an existing RNA polymerase; the synthesis of specific proteins which change the termination, but not the initiation selectivity of the RNA polymerase; and finally, through synthesis of specific polypeptides that change the affinity of the RNA polymerase for specific promoters. Losick and Sonenshein (1969) have characterized a change in the template specificity in the DNA-dependent RNA polymerase of Bacillus subtilis as a result of sporulation. Fukuda et al., (1975, 1977) have observed the presence of two new forms of RNA polymerase in sporulating B. subtilis. The enzymes differed from the vegetative enzyme by the absence of a σ factor, which was replaced by two new polypeptides designated δ^1 and δ^2, respectively. Although both Losick et al., (1969) and Fukuda et al., (1975, 1977) have demonstrated

modification in the RNA polymerase during sporulation of B. subtilis, neither group has been able to demonstrate that the modified enzymes have a greater affinity for sporulation-specific promoters. Some light has been shed on the matter by Doi and Nakayama (1977), who were able to demonstrate that the sporulation-specific RNA polymerase was inhibited to a greater extent with netropsin than the vegetative enzyme, when B. subtilis DNA was used as a template. Netropsin has the capacity to bind to promoter A-T rich regions, and the results suggest that the two enzymes differ in the spectrum of promoter sequences recognized. However, research in this field is made difficult by the presence of proteases in cell extracts, and by the absence of a standardized enzyme purification procedure. The possibility exists of parallel mechanisms in Myxococcus xanthus (Dworkin, 1972) but these remain largely unexplored.

The study of the 3'-termini of bacterial 16S rRNA has proved useful in bacterial systematics. The traditional methods of bacterial classification encounter considerable difficulties when attempts are made to determine relationships beyond the level of genera. This difficulty arises because bacterial genomes have a higher mutation rate than metazoan organisms (Drake, 1970), and because genetic transfer mechanisms in bacteria make possible reticulate evolution (Jones and Sneath, 1970).

The primary structure of 16S rRNAs, on the other hand, has been conserved during the course of bacterial evolution (Woese et al., 1975). Also, the large number of ribosomal genes, which are not all contiguous, make highly unlikely the transfer of ribosomal genes from

one organism to another (Woese et al., 1977). Although the overall primary structure of the 16S rRNAs has been conserved, regions of hypervariability do exist within the molecule (Woese et al., 1977). These qualities of 16S rRNAs make them extremely useful in the elucidation of both distant and close evolutionary relationships.

Bonen and Doolittle (1976) have already employed a method of comparison of 16S rRNA sequences in an attempt to clarify a possible evolutionary relationship between the blue-green bacteria and chloroplasts of eukaryotic algae. Two hypotheses are currently in vogue concerning the relationship between these groups. Stanier (1974) and Margulis (1970) hold that chloroplasts are direct descendants of a procaryote ancestor, while Klein (1970), Allsopp (1969) and, more recently, Cavalier-Smith (1975) suggest that compartmentation of a single photosynthetic cell gave rise to cellular organelles such as nuclei and chloroplasts. The data of Bonen and Doolittle (1976), based on a comparison of 16S ribosomal RNA primary structures suggests that the chloroplasts of the red algae are undeniably of blue-green origin. Moreover, the data confirms the bacterial nature of the blue-greens and their close evolutionary relationship to bacilli. Woese et al., (1977) have employed the same method to study the relationship of the methanogenic bacteria to other procaryotes.

In view of the emerging importance of 16S rRNA primary structure to better understanding of the mechanisms of protein synthesis and bacterial systematics, it is hoped that the elucidiation of the primary structure of 16S rRNA of Myxococcus xanthus will prove useful to systematics of

myxobacteria, and will lead to a better understanding of mechanisms that govern their differentiation.

BIBLIOGRAPHY

Allsopp, A. 1969. Phylogenetic relationships of the prokaryota and the origin of the eukaryotic cell. The New Phytologist. 68:591-612.

Alton, H. T., and H. F. Lodish. 1977. Translational control during the early stages of differentiation of the slime mold Dictyostelium discoideum. Cell. 12:301-310.

Bacon, K., and E. Rosenberg. 1967. Ribonucleic acid synthesis during morphogenesis in Myxococcus xanthus. Journal of Bacteriology. 94:1883-1889.

Bertrand, K., L. Knor, F. Lee, T. Platt, C. L. Squires and C. Yanofsky. 1975. New features of the regulation of the tryptophan operon. Science. 189:22-26.

Bonen, L., and W. F. Doolittle. 1976. Partial sequence of 16S RNA and the phylogeny of blue-green algae and chloroplasts. Nature. 261:669-673.

Bowman, M. C., J. E. Dahlberg, T. Ikemura, J. Konisky, and M. Nomura. 1971. Specific inactivation of 16S ribosomal RNA induced by colicin E3 in vivo. Proceedings of the National Academy of Sciences U.S.A. 68:964-968.

Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Analytical Biochemistry. 1:279-289.

Burchard, P. R., and M. Dworkin. 1966. A bacteriophage for Myxococcus xanthus: isolation, characterization and relation of infectivity to host morphogenesis. Journal of Bacteriology. 91:1303-1305.

Cavalier-Smith, T. 1975. The origin of nuclei and of eukaryotic cells. Nature. 256:463-468.

Ceri, H., and P. Y. Maeba. 1973. Association of a ribonuclease with the 50S ribosomal subunit of Escherichia coli MRE 600. Biochemica et Biophysica Acta. 312:337-348.

Cohen, S. S., and A. Raina. 1967. In "Organizational Biosynthesis", p. 157-182. Ed. H. J. Vogel, J. O. Lampen and V. Bryson. Academic Press, Inc., New York, N.Y.

Cox, R. A. 1970. A spectrophotometric study of the secondary structure of ribonucleic acid isolated from the smaller and larger sub-particles of rabbit reticulocytes. Biochemistry Journal. 117:101-108.

Cox, R. A., H. Pratt, P. Huvos, B. Higginson, and W. Hirst. 1973. A study of the thermal stability of ribosomes and biologically active subribosomal particles. Biochemistry Journal. 134:775-793.

Dahlberg, A. E., E. Lund, N. C. Klejdgaard, C. M. Bowman, and M. Nomura. 1973. Colicin E3 induced cleavage of 16S ribosomal ribonucleic acid; blocking effects of certain antibiotics. Biochemistry. 12:948-950.

Dahlberg, A. E., and J. E. Dahlberg. 1975. Binding of ribosomal protein S1 of Escherichia coli to the 3'-end of 16S rRNA. Proceedings of the National Academy of Science U.S.A. 72:2940-2944.

Darwin, C. 1859. In "The Origin of Species by Means of Natural Selection", p. 133. Ed. J. W. Burrow. Penguin Books Ltd., Harmondsworth, Middlesex, England.

Doi, H. R. 1977. Role of ribonucleic acid polymerase in gene selection in procaryotes. Bacteriological Reviews. 41:568-594.

Drake, J. W. 1974. In "The 24th Symposium of the Society for General Microbiology", p. 41-58. Ed. M. J. Carlile and J. J. Skehel. Cambridge University Press, London.

Dworkin, M. 1962. Nutritional requirements for vegetative growth of Myxococcus xanthus. Journal of Bacteriology. 84:250-252.

Dworkin, M. 1963. Nutritional regulation of morphogenesis in Myxococcus xanthus. Journal of Bacteriology. 86:67-69.

Dworkin, M., and S. M. Gibson. 1964. A system for studying microbial morphogenesis:rapid formation of microcysts in Myxococcus xanthus. Science. 146:243-244.

Dworkin, M., and W. Sadler. 1966. Induction of cellular morphogenesis in Myxococcus xanthus II. Macromolecular synthesis and mechanism of inducer action. Journal of Bacteriology. 91:1520-1525.

Dworkin, M., D. J. Tipper, and D. White. 1968. Peptidoglycan of M. xanthus: structure and relation to morphogenesis. Journal of Bacteriology. 95:2186-2197.

Dworkin, M. 1972. The myxobacteria: new directions in studies of prokaryotic development. Critical Reviews in Microbiology. 1:435-452.

Dworkin, M., and W. J. Wireman. 1975. Morphogenesis and developmental interactions in myxobacteria. Science. 189:516-523.

Ehresmann, C., P. Stiegler, G. A. Mackie, R.A. Zimermann, J. P. Ebel, and P. Fellner. 1975. Primary sequence of the 16S ribosomal RNA of Escherichia coli. Nucleic Acid Research. 2:265-268.

Fellner, P. 1974. In "Ribosomes", p. 169-191. Ed. M. Nomura, A. Tissieres, and P. Lengyel. Cold Spring Harbor Laboratory.

Fortnagel, P., and R. Bergmann. 1973. Alteration of the ribosomal fraction of Bacillus subtilis during sporulation. Biochimica et Biophysica Acta. 299:136-141.

Fortnagel, P., and E. B. Freeze. 1977. Morphological stages of Bacillus subtilis sporulation and resistance to fusidic acid. Journal of General Microbiology. 101:299-306.

Foster, H. A., and H. J. Parish. 1973. Ribosomes, ribosomal subunits and ribosomal proteins from Myxococcus xanthus. Journal of General Microbiology. 75:391-400.

Fukuda, R., G. Keilman, E. McVey and R. H. Doi. 1975. RNA polymerase patterns of sporulating Bacillus subtilis. In "Spores VI", p. 213-220. Ed. P. Gerhardt, R. N. Costilaw, and H. L. Sadoff. The American Society for Microbiology, Washington D.C.

Fukuda, R., and R. H. Doi. 1977. Two polypeptides associated with the ribonucleic acid polymerase core of Bacillus subtilis during sporulation. Journal of Bacteriology. 129:422-432.

Goldberg, M., and J. Steitz. 1974. Cistron specificity of 30S ribosomes heterologously reconstituted with components from Escherichia coli and Bacillus stearothermophilus. Biochemistry. 13:2123-2129.

Gros, F., and M. Grunberg-Manago. 1977. In "Progress in Nucleic Acid Research and Molecular Biology", v. 20 p. 209-284. Ed. W. E. Cohn. Academic Press, Inc. New York, N.Y.

Grover, Y., Y. Pollack, H. Berissi, and M. Revel. 1972. Cistron specific translation control protein in E. Coli. Nature New Biology. 239-16-18.

Grunberg-Manago, M., J. C. Rabinowitz, J. Dondon, J. C. Lebong, F. Gros. 1971. Different classes of initiation factor F3 and their dissociation activity. Federation of European Biochemical Societies Letters. 19:193-199.

Helser, T. L., J. E. Davies and J. E. Dahlberg. 1971. Mechanism of kasugamycin resistance in Escherichia coli. Nature New Biology. 235:6-9.

Hindley, J., and W. H. Staples. 1969. Sequence of a ribosome binding site in bacteriophage Q -RNA. Nature. 224:964-967.

Hunt, J. A. 1965. Terminal-sequence studies of high-molecular weight ribonucleic acid. Biochemistry Journal. 95:541-551.

Hunt, J. A. 1970. Terminal sequence studies of high molecular weight ribonucleic acid. The 3'-termini of rabbit reticulocyte ribosomal RNA. Biochemistry Journal. 120:353-363.

Hunt, J. A. 1973. Terminal sequence studies of high molecular weight ribonucleic acid. The 3'-termini of rabbit globin messenger ribonucleic acid. Biochemistry Journal. 131:315-325.

Johnson, R. Y., and D. White. 1972. Myxospore formation in Myxococcus xanthus: chemical changes in the cell wall during celluar morphogenesis. Journal of Bacteriology. 112:849-855.

Jones, D., and P. H. A. Sneath. 1970. Genetic transfer and bacterial taxonomy. Bacteriological Reviews. 34:40-81.

Kirby, K. S. 1965. Isolation and characterization of ribosomal ribonucleic acid. Biochemistry Journal. 96:266-269.

Klein, R. M. 1970. Relationships between blue-green and red algae. Annals of the New York Academy of Sciences. 175:623-633.

Konisky, J., and M. Nomura. 1967. Interaction of colicins with bacterial cells. II. Specific alteration of Escherichia coli ribosomes induced by colicin E3 in vivo. Journal of Molecular Biology. 26:181-195.

Kurland, C. G. 1960. Molecular characterization of ribonucleic acid from Escherichia coli ribosomes. I. Isolation and molecular weights. Journal of Molecular Biology. 2:83-91.

Lee-Huang, S., and S. Ochoa. 1971. Messenger discriminating species of initiation factor F3. Nature New Biology. 234:236-239.

Lee-Huang, S., and S. Ochoa. 1972. Specific inhibitors of MS2 and late T4 RNA translation in E. coli. Biochemical and Biophysical Research Communications. 49:371-376.

Lee-Huang, S., and S. Ochoa. 1973. Purification and properties of two messenger-discriminating species of E. coli initiation factor 3. Archives of Biochemistry and Biophysics. 156:84-96.

Leffler, S., and W. Szer. 1973. Messenger selection by bacterial ribosomes. Proceedings of the National Academy of Sciences U.S.A. 70:2364-2368.

Leffler, S., and W. Szer. 1974. Purification and properties of initiation factor IF-3 from Caulobacter crescentus. Journal of Biological Chemistry. 249:1458-1464.

Lev, M. 1974. Demonstration of a diffusible fruiting factor in myxobacteria. Nature. 173:501-505.

Lodish, H. F. 1970. Specificity in bacterial protein synthesis: role of initiation factors and ribosomal subunits. Nature. 226:705-707.

Lodish, H. F. 1971. Thermal melting of bacteriophage f2 RNA and the initiation of in vitro protein biosynthesis.

Lodish, H. F. 1976. Translational control of protein synthesis. Annual Review of Biochemistry. 45:39-72.

Losick, R., and A. L. Sonenshein. 1969. Change in the template specificity of RNA polymerase during sporulation of Bacillus subtilis. Nature. 224:35-37.

Maizels, N. 1974. Escherichia coli lactose operon ribosome binding site. Nature. 249:647-649.

Margulis, L. 1970. Origin of eukaryotic cells. Yale University Press, New Haven, Connecticut.

McCurdy, D. H. 1969. Light and electron microscopic studies on the fruiting bodies of Chondromyces crocatus. Archives of Microbiology. 65:380-384.

McCurdy, D. H. 1974. In "Bergey's Manual of Determinative Bacteriology", p. 76-98. Ed. S. T. Cowan, J. G. Holt, J. Liston, R. E. Murray, C. F. Niven, A. W. Ravin, and R. Y. Stanier. The Williams and Wilkins Co., Baltimore.

McMahon, D. 1973. A cell-contract model for cellular position determination in development. Proceedings of the National Academy of Science U.S.A. 72:2940:2944.

Miller, Z., E. R. Varmus, H. E. Parks, J. S. Perlman, and I. Pastan 1971. Regulation of gal messenger ribonucleic acid synthesis in Escherichia coli by 3'-5'-cyclic adenosine monophosphate. Journal of Biological Chemistry. 246:2848-2903.

Newell, P. C., M. Longlands and M. Sussman. 1971. Control of enzyme synthesis by cellular interaction during development of the cellular slime mold Dictyostelium discoideum. Journal of Molecular Biology. 58:541-554.

Nomura, M., W. A. Held, and W. R. Gette. 1974. Role of 16S ribosomal ribonucleic acid and the 30S ribosomal protein S12 in the initiation of natural messenger ribonucleic acid translation. Biochemistry. 13:2115-2122.

Parish, H. J., R. K. Wedgewood, and D. G. Herries. 1976. Morphogenesis in Myxococcus xanthus and Myxococcus virescens. Archives of Microbiology. 107:343-351.

Peacock, A. C., and C. W. Dingman. 1968. Analytical studies on nuclear ribonucleic acid using polyacrylamide gel electrophoresis. Biochemistry. 7:659-668.

Perry, P. R. 1976. Processing of RNA. Annual Review of Biochemistry. 45:605-629.

Pollack, Y., Y. Graner, H. Aviv, and M. Revel. 1970. Fractionation of translation initiation factor B (F3) into cistron-specific species. Federation of European Biochemical Societies Letters. 9:213-217.

RajBhandary, A. L., and S. H. Chang. 1968. Yeast phenylalanine transfer ribonucleic acid: partial digestion with ribonuclease T1 and derivation of the total primary structure. Journal of Biological Chemistry. 243:598-601.

Ramsey, W. S., and M. Dworkin. 1968. Microcyst germination in Myxococcus xanthus. Journal of Bacteriology. 95:2249-2257.

Reichenbach, H. 1965. Schwarmentwiklung and Morphogense bei Myxobacterien Archangium, Myxococcus, Chondrococcus, Chondromyces. Film #C-893, Institut, F. D. Wissench, Film, Gottingen, West Germany.

Reichenbach, H., and M. Dworkin. 1969. Studies on Stigmatella aurantiaca (Myxobacterales.) Journal of General Microbiology. 58:3-14.

Rosenberg, E. M., M. Katarski, and P. Gottlieb. 1967. Deoxyribonucleic acid synthesis during exponential growth and microcyst formation in Myxococcus xanthus. Journal of Bacteriology. 93:1402-1408.

Rosenberg, E., and S. S. Witkin. 1970. Induction of morphogenesis by methionine starvation in Myxococcus xanthus: polyamine control. Journal of Bacteriology. 103:641-649.

Schiff, N., M. Miller, and A. Wahba. 1974. Purification and properties of chain initiation factor 3 from T4-infected and uninfected Escherichia coli MRE 600. Journal of Biological Chemistry. 249:3797-3802.

Schleif, R. 1971. L-Arabinose operon messenger of Escherichia coli: its inducibility and translation efficiency relative to lactose operon messenger. Journal of Molecular Biology. 61:275-279.

Schroeder, E., J. McKibbin, L. S. Mitchell, and N. R. Pace. 1977. Mode of degradation of precursor-specific ribonucleic acid fragments by Bacillus subtilis. Journal of Bacteriology. 130:1000-1009.

Shine. J., and L. Dalgarno. 1973. Conserved terminal sequence in 18S rRNA may represent terminator anticodens. Nature New Biology. 245:261-262.

Shine, J., and L. Dalgarno. 1974. The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proceedings of the National Academy of Sciences, U.S.A. 71:1342-1346.

Shine, J., and L. Dalgarno. 1975. Determination of cistron specificity in bacterial ribosomes. Nature. 254:34-38.

Spermulli, L., M. Haralson and J. Ravel. 1974. Effects of T4 infection on initiation of protein synthesis and messenger specificity of initiation factor 3. Archives of Biochemistry and Biophysics. 165:581-587.

Sprague, K. U., J. A. Steitz, R. M. Grenley, and C. E. Stocking. 1977. 3'-terminal sequences of 16S rRNA do not explain translational specificity differences between E. Coli and B. stearothermophilus ribosomes. Nature. 267:462-465.

Stanier, R. Y. 1973. In "The Twenty-fourth Symposium of the Society for General Microbiology", p. 219-239. Cambridge University Press.

Stanley, W. M. Jr., and R. M. Boch. 1965. Isolation and physiological properties of the ribosomal ribonucleic acid of Escherichia coli. Biochemistry. 4:1302-1311.

Steinschneider, A., and H. Fraenkel-Conrat. 1966. Studies of nucleotide sequences in tobacco mosaic virus ribonucleic acid. VI. Use of aniline in stepwise degradation. Biochemistry. 5:2735-2743.

Steitz, J. A. 1969. Polypeptide chain initiation: nucleotide sequences of the three ribosomal binding sites in bacteriophage R17 RNA. Nature. 224:953-964.

Steitz, J. A. 1973. Specific recognition of non-initiator region in RNA bacteriophage messengers by ribosomes of Bacillus stearothermophilus. Journal of Molecular Biology. 73:1-16.

Steitz, J. A., and K. Jakes. 1975. How ribosomes select initiator regions in mRNA: base pair formation between the 3'-terminus of 16S rRNA and the mRNA during initiation of protein synthesis in Escherichia coli. Proceedings of the National Academy of Science U.S.A. 72:4734-4738.

Stoffler, G. 1974. In "Ribosomes", p. 615-667. Ed. M. Nomura, A. Tissières, P. Lengyel. Cold Spring Harbor Laboratory.

Szer, W., and J. Brenowitz. 1970. Translation of MS2 by ribosomes from different species. Biochemical and Biophysical Research Communications. 38:1154-1160.

Tener, G. M. 1967. In "Methods of Enzymology", v. 12, part C, p. 398-401. Ed. L. Grossman and K. Moldave. Academic Press, Inc. New York. N.Y.

Thaxter, R. 1892. On the Myxobacteraceae, a new order of Schizomycetes. Botanical Gazette. 17:389-404.

Traut, R. R., R. L. Heimark, T-T. Sun, J. W. B. Hershey and A. Bollen. 1974. In "Ribosomes", p. 271-308. Ed. M. Nomura, A. Tissieres, and P. Lengyel. Cold Spring Harbor Laboratory.

Varmus, H. E., R. Perlman and I. Pastan. 1970. Regulation of lac messenger ribonucleic acid synthesis by cyclic adenosine 3',5'-monophosphate and glucose. Journal of Biological Chemistry. 245:2259-2267.

Voelz, H. 1967. The physical organization of the cytoplasm in Myxococcus xanthus and the fine structure of its components. Archives of Microbiology. 57:181-186.

Voelz, H., and H. Reichenbach. 1969. Fine structure of fruiting bodies of Stigmatella aurantiaca. Journal of Bacteriology. 99:2249-2257.

Watson, B. F., and M. Dworkin. 1968. Intermediary metabolism of vegetative cells and microcysts of Myxococcus xanthus. Journal of Bacteriology. 96:1473-1645.

Weber, H., M. A. Billeter, S. Kahone, C. Weissmann, J. Hindley, and A. Porter. 1972. Molecular basis for repressor activity of Qβ replicase. Nature New Biology. 237:166-169.

Woese, C. R., G. E. Fox, L. Jablen, T. Uchida, L. Bonen, K. Pechman, B. J. Lewis, and D. Stahl. 1975. Conservation of primary structure in 16S ribosomal RNA. Nature. 254:83-86.

Woese, C. R., P. R. Kenneth, and G. E. Fox. 1977. Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to prokaryote systematics. International Journal of Systematic Bacteriology. 27:44-57.

Woese, C. R., G. E. Fox, J. M. Linda, E. W. Balch, and R. S. Wolfe. 1977. Classification of methanogenic bacteria by 16S ribosomal RNA characterization. Proceedings of the National Academy of Sciences U.S.A. 10:4533-4541.

Züsman, D., and E. Rosenberg. 1968. Deoxyribonucleic acid synthesis during microcyst germination in Myxococcus xanthus. Journal of Bacteriology. 96:981-986.