***In vitro* Transcription and Translation Directed by *Caulobacter crescentus* CB15 Nucleoids**

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(Received 10 March 1981)

Envelope-associated nucleoids isolated from *Caulobacter crescentus* contained DNA-dependent RNA polymerase and nascent RNA. In the presence of nucleotide substrates, and under suitable conditions, RNA chain elongation took place at similar rates on both fast sedimenting (FS) and slow sedimenting (SS) nucleoids. Reinitiation of transcription did not occur *in vitro* except when exogenous holoenzyme was added to the assay system. Three major classes of RNA were synthesized *in vitro*. The two larger species were heterodisperse and had electrophoretic mobilities equivalent to 16–20S and 23–35S. The third class contained RNA in the region of 3–4S. The high molecular weight components were absent from RNA synthesized on FS nucleoid templates whereas the 3–4S fraction was much increased.

Caulobacter crescentus nucleoids were also examined for the presence of mRNA in a protein-synthesizing system derived from *Escherichia coli* cells. The activity of heterologous mRNA was detectable only after the conversion of *E. coli* cell lysates into an mRNA-dependent translation system by treatment with micrococcal nuclease. Incorporation of [³⁵S]methionine into trichloroacetic acid-insoluble material reflected synthesis of *bona fide* template-specific polypeptides by several criteria showing that SS and FS envelope-associated nucleoids contained biologically active mRNAs. Since the nucleoids were transcriptionally inactive during protein synthesis, the assay reflected the presence of mRNAs produced at different stages in the *Caulobacter* cell cycle. The translational capacity of the nucleoids increased markedly after transcription indicating that some RNA synthesized *in vitro* was mRNA. The specific activity of transcribed nucleoids as templates for protein synthesis supported the contention that the predominant RNAs synthesized *in vitro* were rRNAs and their precursors.

INTRODUCTION

Morphogenesis and differentiation in prokaryotes must involve control systems which regulate the timing of each cell cycle event and coordinate them to produce structural and metabolic changes in the differentiating cells. The study of the control of transcription and translation requires a suitable *in vitro* system in which the control systems, as they exist in the living cell, have not been greatly perturbed or disorganized. At present, bacterial nucleoids provide the best available model system for this purpose and have been studied in detail in *Escherichia coli* (Giorno *et al.*, 1975; Pettijohn, 1976) and *Bacillus subtilis* (Guillen *et al.*, 1977; Hirschbein *et al.*, 1976).

In previous papers we described the isolation of two types of nucleoids from heterogeneous populations of exponentially growing *Caulobacter crescentus* CB15 cells (Swoboda & Dow,

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1979; Swoboda *et al.*, 1982). Fast sedimenting (FS) and slow sedimenting (SS) envelope-associated nucleoids were separated by density gradient centrifugation and characterized, with respect to the cell types from which they were derived, by cell envelope morphology. It was hypothesized that the SS nucleoids were derived from cells undergoing DNA replication while FS nucleoids were derived from cells (predominantly swarmer cells) in which there was no DNA synthesis. This was not, however, thought to be the major reason for the difference in sedimentation coefficients (5600S and 7100S); the difference was thought to be due to the enhanced protein to DNA ratio of the FS nucleoids. In this paper we report *in vitro* transcription and translation studies using both types of nucleoids.

METHODS

Chemicals. Trizma base, nucleoside triphosphates, rifampicin, *E. coli* RNA polymerase [specific activity 900 units (mg protein)⁻¹], pancreatic ribonuclease A and bovine serum albumin were purchased from Sigma. Deoxyribonuclease I (RNAase-free) was obtained from Worthington. Micrococcal nuclease and phosphoenolpyruvate were purchased from Boehringer. [5-³H]Uridine 5'-triphosphate (15 Ci mmol⁻¹, 0.56 TBq mmol⁻¹), L-[³⁵S]methionine (600 Ci mmol⁻¹, 22 TBq mmol⁻¹), adenosine 5'-[γ -³²P]triphosphate (3000 Ci mmol⁻¹, 111 TBq mmol⁻¹) and uridine 5'-[α -³²P]triphosphate (400 Ci mmol⁻¹, 14.8 TBq mmol⁻¹) were obtained from Amersham. All other chemicals were purchased from BDH, unless otherwise stated.

Bacterial strains and growth conditions. *Caulobacter crescentus* CB15 was grown at 30 °C in Hunter's minimal medium (Swoboda *et al.*, 1982). *Escherichia coli* strains MRE600 and B were grown at 37 °C in YK medium (Hagen & Young, 1973). *Bacillus licheniformis* A5 was grown at 37 °C in B medium (Vitkovic & Sadoff, 1977).

Preparation of templates. Fast sedimenting (FS) and slow sedimenting (SS) envelope-associated nucleoids were isolated from heterogeneous populations of exponentially growing *C. crescentus* cells as described by Swoboda *et al.* (1982). Nucleoids were used in transcription and translation experiments immediately after isolation. RNA synthesized *in vitro* was translated without purification immediately after transcription. RNA standards were prepared as described by Swoboda *et al.* (1982), but *E. coli* and *B. licheniformis* RNA for translation studies was extracted from exponentially growing cells as described by Hagen & Young (1973).

In vitro transcription studies

(i) ***In vitro* RNA synthesis.** Reaction mixtures contained the following constituents in a final volume of 0.2 ml: 1 mM each of ATP, CTP, GTP; 0.05 mM-UTP; 1 μ Ci [³H]UTP ml⁻¹, or 1 μ Ci [³²P]ATP ml⁻¹; 12.5 mM-MgCl₂; 100 mM-Tris/HCl (pH 7.5 at 20 °C); 7.5 mM-2-mercaptoethanol; 1 mM-spermidine hydrochloride; specified concentrations of nucleoids; 5 μ g of *E. coli* DNA-dependent RNA polymerase holoenzyme. Reactions were started by transferring the assay mixtures from 0 °C to 37 °C. Duplicate 20 μ l samples were removed at regular intervals, spotted on to Whatman 3MM filter discs, immersed immediately into ice-cold 5% (w/v) trichloroacetic acid (TCA) containing 0.1% (w/v) tetra-sodium pyrophosphate and left for 10 min. Filters were washed twice in the 5% TCA/0.1% tetra-sodium pyrophosphate solution and twice in ethanol at 10 min intervals before being dried and counted in 3 ml of Triton/toluene scintillant in a Packard Tri-Carb liquid scintillation spectrometer.

(ii) **Purification of transcripts from the reaction mixture.** Protein and labelled triphosphates were removed from the RNA by phenol extraction (Kirby, 1965). The transcription assay mixture was scaled up to 2 ml. At 5 min and 40 min after the initiation of RNA synthesis, 1 ml of the mixture was diluted to 2 ml with water and added to 2 ml of double-strength Kirby mixture [50 mM-KCl, 1% (w/v) tri-isopropyl naphthalenesulphonate, 6% (w/v) 4-aminosalicylic acid (sodium salt), 10 mM-Tris/HCl, pH 7.6]. Total nucleic acid was extracted with 8 ml of a phenol/*m*-cresol/8-hydroxyquinoline mixture (500:70:0.5, by wt). After shaking for 2 min the phases were separated by centrifugation at 3500 g for 10 min. The nucleic acid was re-extracted from the aqueous phase two or three times more and precipitated overnight with 2 vol. ethanol after the addition of 100 μ g of *E. coli* RNA to act as carrier during the alcohol precipitation. The nucleic acid was recovered by centrifugation (3500 g at 4 °C for 15 min) and washed twice in a solution containing 70% (v/v) ethanol, 0.5% (w/v) sodium dodecyl sulphate (SDS) and 0.1 M-NaCl, before checking the purity by scanning with a Pye Unicam SP800 spectrophotometer. Washing was repeated until there was no contamination by either phenol or carbohydrate. The resulting total nucleic acid was washed twice in 70% ethanol to remove the SDS. DNA was then removed by treating with 100 μ g DNAase I (RNAase-free) at room temperature for 30 min, and the RNA was re-extracted with the phenol mixture. RNA was precipitated with 2 vol. ethanol at -20 °C overnight, recovered by centrifugation, washed twice with a solution containing 70% ethanol, 0.1% SDS and 0.1 M-NaCl, resuspended in 0.1 M-sodium acetate buffer, pH 6.2, containing 0.5% SDS, and reprecipitated with 2 vol. ethanol. RNA was stored in 2 vol. ethanol at -20 °C until required. As a control, during DNAase treatment, duplicate samples to the above were treated with 100 μ g RNAase A.

To determine the level of incorporation of ^3H or ^{32}P into the RNA, 10 μl of a solution of known concentration of labelled RNA dissolved in the SDS/sodium acetate buffer, pH 6.2, was spotted on to a Whatman GF/A glass-fibre disc, washed, dried and the radioactive content was determined.

(iii) *Polyacrylamide gel electrophoresis of transcripts.* The size of the ^3H -labelled RNA synthesized *in vitro* was determined by electrophoresis on 2.4% (w/v) polyacrylamide gels, as described by Loening (1967, 1969). In view of the quenching of ^3H counts by dried polyacrylamide gel slices, a bisacrylamide cross-linked gel, which can be dissolved in hydrogen peroxide, was used. Electrophoresis was at room temperature at a constant current of 5mA per gel. After electrophoresis, the gels were transferred to a rectangular quartz cell and scanned using a Gilford spectrometer. At the end of the scan, the length of the gel in the cell was measured and the gel was frozen in an aluminium trough on powdered dry ice. After freezing, each gel was sequentially sliced into 1 mm slices. The slices were dissolved in 0.3 ml 30% hydrogen peroxide by incubating at 60 °C in sealed scintillation vials overnight. After cooling, 3 ml of Triton/toluene scintillant was added to each vial. The amount of ^3H in each gel slice was determined and the RNA profile obtained was related to the ultraviolet scan.

In vitro translation studies

(i) *Preparation of cell-free extract.* An S30 extract was prepared from *E. coli* MRE600 cells as described by Hagen & Young (1973). In preliminary experiments the extract was preincubated for 20 min at 37 °C in the presence of 4 μg DNAase 1 ml^{-1} . This failed to lower the incorporation of [^{35}S]methionine into TCA-insoluble material sufficiently. Subsequently, we employed the method of Pelham & Jackson (1976). DNAase was replaced by 10 μg micrococcal nuclease ml^{-1} in the presence of 1 mM- CaCl_2 . The digestion of DNA and RNA was terminated by the addition of EGTA to 2 mM final concentration. The S30 extract was then used for protein synthesis.

(ii) *Cell-free protein synthesis.* The reaction mixture for protein synthesis (usually 100 μl) contained 0.2 mM-GTP, 3.0 mM-ATP, 5.0 mM-phosphoenolpyruvate, 2.4 mM-dithiothreitol (Cleland's reagent), 60 mM-Tris/HCl, pH 7.8, 8.0 mM-magnesium acetate, 110 mM-ammonium acetate, 0.8 mM-2-mercaptoethanol (from the S30 extract), 0.5 μM -leucine, 0.05 mM-tyrosine, 0.11 mM of the other 17 amino acids, 3.8 mM-polyethylene glycol, 5 mg S30 protein ml^{-1} , RNA template and [^{35}S]methionine as indicated.

(iii) *Estimation of [^{35}S]methionine incorporation.* Bovine serum albumin was added to samples to achieve a final concentration of 2.5 mg ml^{-1} and proteins were precipitated with 1 ml 10% TCA for 10 min at 4 °C. Precipitated material was recovered by centrifuging, resuspended in 0.5 ml 0.2 M-NaOH, incubated for 15 min at 45 °C and reprecipitated with 1.5 ml 10% TCA. The precipitate was recovered by filtration through Whatman GF/C glass-fibre discs, washed with 15 ml 5% (w/v) cold TCA and 5 ml 90% ethanol and dried. Counting was performed in a toluene-based scintillant by liquid scintillation spectrophotometry. Efficiency of counting was approximately 70% for [^{35}S]methionine.

(iv) *Preparation and electrophoresis of translation products.* The reaction products of cell-free protein synthesis were processed essentially as described by Hopper *et al.* (1975). To 0.8 ml of each sample, 1.0 ml 90% (v/v) aqueous acetone was added to precipitate labelled proteins. Samples were mixed and incubated at 4 °C for 30 min. The precipitate was pelleted by centrifugation at 4 °C. The supernatant was discarded and residual acetone was removed by evaporation. Cracking buffer (0.8 ml; Studier, 1973) was added, the samples were boiled for 3 min and then stored at -20 °C. SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed as described by Studier (1973) and Lagenaur & Agabian (1976).

RESULTS

In vitro transcription studies using nucleoids as templates

Template capacity of the fast sedimenting and slow sedimenting envelope-associated nucleoids. The reaction mixture for assay of RNA synthesis contained nucleoids, DNA-dependent RNA polymerase, buffer, monovalent and divalent cations and nucleoside triphosphates in the optimal concentrations to give the maximum level of transcription. Spermidine was added to stimulate RNA synthesis *in vitro* and prevent first order inhibition of RNA polymerase (Loening, 1967; Fuchs *et al.*, 1967). Spermidine also stabilizes the condensed state of the isolated nucleoids increasing their transcriptional capacity *in vitro* (Flink & Pettijohn, 1975; Giorno *et al.*, 1975). 2-Mercaptoethanol was added to maintain the DNA-dependent RNA polymerase in a stable, reduced form. RNA synthesis, expressed as the incorporation of [^3H]UMP into TCA-precipitable material, as a function of time is shown in Fig. 1. When nucleoids were incubated in the reaction mixture containing all of the substrates except exogenous DNA-dependent RNA polymerase, some RNA synthesis was

Fig. 1. *In vitro* RNA synthesis. The kinetics of incorporation of [^3H]UTP by 10 μg of SS or FS envelope-associated nucleoids or calf thymus DNA, with and without the addition of *E. coli* RNA polymerase, was determined as described in Methods. Samples (20 μl) were removed at intervals and the ^3H incorporated into RNA was determined. SS nucleoids ($\blacktriangle$); SS nucleoids plus *E. coli* RNA polymerase ($\triangle$); FS nucleoids ($\circ$); FS nucleoids plus *E. coli* RNA polymerase ($\bullet$); Calf thymus DNA ($\blacksquare$); Calf thymus DNA plus *E. coli* RNA polymerase ($\square$).

Table 1. *Characterization of incorporation of [^3H]UTP by SS envelope-associated nucleoids of *C. crescentus**

Assays for incorporation (40 min incubation) were performed according to the procedure in Methods. Where indicated, rifampicin was added at 200 $\mu\text{g ml}^{-1}$, actinomycin D at 50 $\mu\text{g ml}^{-1}$ and RNAase I at 1 $\mu\text{g ml}^{-1}$.

Constituents	Relative incorporation (%)
Standard assay mixture	100
+ Rifampicin	20
+ Actinomycin D	19
– RNA polymerase	21
– RNA polymerase + rifampicin	21
– RNA polymerase + rifampicin after 5 min	21
– ATP, GTP or CTP	10
– ATP, GTP and CTP	<1
+ RNAase	1

detected, indicating that both the FS and SS envelope-associated nucleoids contained active endogenous DNA-dependent RNA polymerase. When exogenous *E. coli* RNA polymerase was added to the reaction mixture, with DNA as the limiting factor, the rate of RNA synthesis increased 3- to 4-fold. Within experimental error, the rates of RNA synthesis by equal concentrations of FS and SS envelope-associated nucleoids were almost identical. Synthesis was initiated immediately at the incubation temperature and continued for over 60 min. The rate of synthesis remained linear and constant for 30 min. Three control experiments confirmed that the measurements represented *in vitro* RNA synthesis (Table 1): (i) the omission of either ATP or CTP or GTP reduced incorporation by 90%; (ii) no TCA-insoluble radioactive material was recovered after treatment with pancreatic RNAase A; (iii) addition of actinomycin D or rifampicin (see also Fig. 3) reduced incorporation by 80%.

The sedimentation coefficients of the FS and SS nucleoids showed no appreciable change during the time course of the transcription experiments.

Fig. 2. Effect of salt concentration on transcription. Assays were performed by the standard procedure described in Methods except that the NaCl concentration was varied as indicated. SS envelope-associated nucleoids (□); FS envelope-associated nucleoids (▲); calf thymus DNA (●).

Fig. 3. Effect of rifampicin on RNA synthesis by FS and SS envelope-associated nucleoids in the presence and absence of *E. coli* RNA polymerase. Rifampicin was added to 10 μl assay mixtures to give 200 $\mu\text{g ml}^{-1}$. (a, c) Transcription of FS (a) and SS (c) nucleoids in the presence of *E. coli* RNA polymerase without rifampicin (○) and with rifampicin (●). (b, d) Transcription of FS (b) and SS (d) nucleoids by endogenous RNA polymerase without rifampicin (△) and with rifampicin added at zero time (■) and after 5 min incubation (□).

In the presence of exogenous *E. coli* DNA-dependent RNA polymerase both FS and SS envelope-associated nucleoids were more efficient templates than purified calf thymus DNA (Fig. 1).

Effect of salt and temperature on transcription. High ionic conditions were not conducive to maximal RNA synthesis. When both FS and SS envelope-associated nucleoids were incubated with increasing concentrations of NaCl in the assay mixture, the rate of RNA synthesis decreased until at 200 mM-NaCl no synthesis occurred. Maximal synthesis was obtained when the salt concentration was 10 mM (Fig. 2). Transcription of calf thymus DNA

Fig. 4. Kinetics of incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ into RNA synthesized from FS and SS envelope-associated nucleoids. Assays were performed as described in Methods in the presence of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($1\ \mu\text{Ci ml}^{-1}$). Incorporation from $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ by FS (■) and SS (●) nucleoids; incorporation from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by FS (□) and SS (○) nucleoids.

was affected differently by the salt concentration (Fig. 2). For both nucleoids and calf thymus DNA, *in vitro* RNA synthesis was maximal at 37°C .

Nature of RNA synthesis in vitro. Transcription by exogenous polymerase of both FS and SS envelope-associated nucleoids was rifampicin-sensitive but the antibiotic had no effect on endogenous RNA synthesis (Fig. 3). In the presence of rifampicin, the concentration of RNA synthesized by exogenous DNA-dependent RNA polymerase dropped to the endogenous level. Since rifampicin is an inhibitor of chain initiation but not chain growth, these results suggested that reinitiation of transcription by endogenous RNA polymerase did not occur in either the FS or SS envelope-associated nucleoids, but did occur once exogenous RNA polymerase had been added.

Reinitiation of RNA chains was also followed by measuring the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into TCA-insoluble RNA. The level of incorporation was very low (40 min incubation time) when compared with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ incorporation in the control experiment (Fig. 4).

Analysis of RNA synthesized in vitro. The RNA products synthesized on the isolated nucleoids by incubation for 5 min and 40 min were purified and analysed by polyacrylamide gel electrophoresis. (From data on the kinetics of RNA synthesis, 40 min was sufficient time to complete chain elongation.) Polyacrylamide disc gels (Fig. 5) indicated that both FS and SS envelope-associated nucleoids synthesized homogeneous low molecular weight transcripts. In addition, the SS envelope-associated nucleoids synthesized two larger molecular weight species which appeared as heterogeneous peaks. Traces of these were also apparent during transcription of FS nucleoids. Comparison of the *in vitro* transcripts with *E. coli* marker RNAs indicated that the transcripts with the greatest electrophoretic mobility, which were synthesized by both FS and SS envelope-associated nucleoids, had a sedimentation coefficient of 3.2S ($\pm 0.5\text{S}$). The larger of the two high molecular weight species had a sedimentation coefficient ranging from approximately 23S to 35S , while the smaller component had a sedimentation coefficient ranging from 16S to 20S ($\pm 0.5\text{S}$) (Pettijohn *et al.*, 1970*a, b*). The heterogeneity of these two peaks could be explained by assuming that the larger species, besides containing mature 23S rRNA, also contained the precursor molecules of 30S and 25S RNA, while the smaller species contained not only the 16S rRNA but also the 17.5S precursor. In prokaryotes, the 30S precursor is rapidly cleaved during the course of its synthesis to 25S and 17.5S RNA fragments, which in turn are further processed to the mature 23S and 16S rRNA (Nikolev *et al.*, 1973, 1974). The presence of the 3–4S RNA may be

Fig. 5. Polyacrylamide gel electrophoresis of RNA synthesized *in vitro*. Transcription (40 min), purification and sizing of transcripts was carried out as described in Methods. Unlabelled marker RNAs were extracted by the method of Kirby (1965). Electrophoresis was carried out in 2.4% polyacrylamide gels at 5 mA per gel and at a constant voltage for 3–4 h. Radioactivity in transcripts synthesized on FS (a) and SS (b) envelope-associated nucleoids (—); A_{260} of *E. coli* rRNA and tRNA (---).

explained as being due to insufficient polymerization or frequent chain termination, to degradation of RNA, to the release of the 'spacer' regions, by endonucleolytic degradation, from the ribosomal precursor RNA molecules, or to tRNA. The high background and the incomplete resolution of the peaks could be due, at least in part, to the synthesis of mRNA on the nucleoids.

Nucleoid-directed cell-free protein synthesis

Modification of S30 extract. An S30 extract prepared from *E. coli* is saturated with endogenous mRNA and is capable of *in vitro* transcription of endogenous DNA (Zubay, 1973). Additional mRNA will, therefore, only be translated to the extent that it competes with the endogenous mRNA. In order to measure the stimulation by exogenously added mRNA, and to detect the products synthesized, the S30 extract was preincubated in the presence of DNAase and the absence of labelled amino acid. The aim was to run off endogenous mRNA from the existing polyribosomes and to degrade any endogenous mRNA and DNA present. However, this procedure proved to be insufficient to lower the activity of endogenous mRNA and allow the detection of nucleoid activity. Table 2 shows that the incorporation directed by endogenous mRNA ('background') was much lower after treatment with micrococcal nuclease than with DNAase. Thus, preincubation of the S30 extract with Ca^{2+} -dependent micrococcal nuclease permitted detection of amino acid incorporation directed by *E. coli* B and *C. crescentus* mRNA. Similar results have been obtained with coliphage MS2 and *B. licheniformis* A5 mRNA. Background incorporation was reduced 5-fold by the nuclease compared with DNAase treatment and consequently stimulation by the SS nucleoids increased from 1.1- to 3.8-fold (Table 2).

Table 2. *Effect of preincubation of E. coli S30 extract on amino acid incorporation into TCA-insoluble polypeptides*

The techniques used in preincubating S30 extract with DNAase or micrococcal nuclease are described in Methods. Reaction mixtures (100 μ l) contained preincubated S30 extract alone or with *E. coli* B exogenous RNA (0.5 μ g) or *C. crescentus* SS nucleoids (containing 0.5 μ g RNA), and [35 S]methionine (specific activity 100 Ci mmol $^{-1}$, final concentration 100 pmol ml $^{-1}$). The mixtures were incubated at 37 °C for 20 min, and the incorporation of [35 S]methionine into TCA-insoluble polypeptides was measured as pmol per ml reaction mixture.

Template	Preincubation with DNAase		Preincubation with nuclease	
	Incorporation (pmol ml $^{-1}$)	Stimulation (-fold)	Incorporation (pmol ml $^{-1}$)	Stimulation (-fold)
Residual endogenous RNA in <i>E. coli</i> S30 extract	0.32	—	0.07	—
+ <i>E. coli</i> exogenous RNA	0.71	2.2	0.35	5.0
+ <i>C. crescentus</i> SS nucleoids	0.35	1.1	0.27	3.8

Table 3. *Effect of preincubation of C. crescentus nucleoids in a transcription assay mixture on in vitro translation*

Reaction mixtures (100 μ l) were incubated at 37 °C for 20 min after addition of [35 S]methionine (specific activity 1205 Ci mmol $^{-1}$, final concentration 6 pmol ml $^{-1}$). Where indicated, isolated nucleoids were preincubated in an *in vitro* transcription assay mixture for 40 min. Isolated FS and SS nucleoids contain about the same amount of RNA, i.e. 25–27% (w/w) of the total cellular DNA (Swoboda *et al.*, 1982), and so the results in the table are directly comparable.

Template	[35 S]Methionine incorporated in 20 min [pmol (μ g RNA) $^{-1}$]	
	FS nucleoids	SS nucleoids
Isolated nucleoids	0.012	0.040
Isolated nucleoids preincubated in transcription assay mixture	0.141	0.096

Cell-free protein synthesis. The incorporation of [35 S]methionine into TCA-insoluble products reflects *de novo* protein synthesis programme by exogenous templates. The incorporation was abolished by pretreatment with RNAase or by addition of trypsin after the translation. Omission of leucine and/or tyrosine from the reaction mixture led to a decrease in incorporation of labelled amino acid. SDS-PAGE of the translation products showed that [35 S]methionine was incorporated in the presence of nucleoids into several major polypeptides, whereas no observable proteins were synthesized by controls. Although our results are preliminary, a comparison of the proteins synthesized in the presence of *E. coli* B mRNA and *C. crescentus* SS nucleoid RNA has shown them to be different in number, molecular weight and relative amounts.

Specific template activity of the nucleoids. Nucleoid-associated mRNAs were quantified by measuring the amount of labelled amino acid incorporated into protein per μ g of total RNA present initially. The results indicated that both SS and FS nucleoids contained biologically active mRNAs (Table 3). In some experiments, nucleoids were transferred into the transcription reaction mixtures immediately after isolation. Under the conditions described earlier, RNA synthesis occurred and was monitored by [3 H]UTP incorporation. The reaction was terminated after 40 min by chilling to 4 °C. The appropriate samples were then removed from duplicate tubes containing unlabelled UTP and transferred into protein-synthesizing

assay mixtures. Translation of *in vitro* and *in vivo* synthesized mRNAs commenced when the temperature was increased to 37 °C upon the addition of [³⁵S]methionine. The translational capacity of the nucleoids increased markedly after transcription, indicating that some RNA synthesized *in vitro* was mRNA (Table 3). The specific activity of FS nucleoids as a template for protein synthesis increased approximately 10-fold after *in vitro* transcription and was higher than the specific activity of transcribed SS nucleoids.

DISCUSSION

The first part of this study was concerned with comparing *in vitro* transcription by FS and SS envelope-associated nucleoids of *Caulobacter crescentus* CB15. The isolation of nucleoids in high ionic strength buffers (Giorno *et al.*, 1975) stabilizes their structure but is not compatible with RNA synthesis. For this reason nucleoids were isolated in low salt medium in the presence of spermidine (Stonington & Pettijohn, 1971) and Mg²⁺. This procedure not only stabilizes the nucleoids but gives optimal conditions for RNA synthesis.

SS and FS nucleoids isolated in this manner are transcriptionally active. They contain endogenous RNA polymerase which can elongate *in vitro* RNA transcripts initiated *in vivo*. Experiments with rifampicin, however, indicated that no reinitiation occurred with endogenous polymerase under *in vitro* conditions. This is almost certainly caused by the loss of the σ factor and/or the holoenzyme on isolation. Similar results have been obtained with *Bacillus subtilis* nucleoids (Guillen *et al.*, 1977).

On addition of *E. coli* RNA polymerase (holoenzyme) to *C. crescentus* nucleoids, transcriptional activity increased 5-fold over the endogenous level. This increased activity was rifampicin-sensitive indicating that reinitiation had occurred. It would have been preferable to use exogenous *C. crescentus* RNA polymerase; however, Amemiya & Shapiro (1977) have established that the *C. crescentus* and *E. coli* core RNA polymerases and σ subunits are functionally analogous, i.e. *C. crescentus* core RNA polymerase can be used in conjunction with *E. coli* σ factor to transcribe *E. coli* templates and vice versa.

A very low rate of incorporation of phosphate from [γ -³²P]ATP into RNA transcripts was apparent when compared with that of phosphate from [α -³²P]UTP. However, the ratio of terminal triphosphate to internucleotide phosphates is very low in RNA, and so these results are not inconsistent with reinitiation occurring as predicted from the experiments with rifampicin.

FS and SS nucleoids were more active templates directing twice the rate of transcription compared with an equivalent weight of purified calf thymus DNA in a low salt assay system. Nucleoid transcription also differed from that of naked DNA in its kinetics and sensitivity to high salt concentrations. This suggests that the tertiary structure of the folded DNA in nucleoids (Swoboda *et al.*, 1982), together with its association with DNA-binding proteins (Haselkorn & Rouvière-Yaniv, 1976; Rouvière-Yaniv *et al.*, 1979; Lathe *et al.*, 1980), provides a more efficient controlled template for transcription than naked DNA.

Although the rate of RNA synthesis *in vitro* from both types of nucleoids was similar, the RNA species synthesized differed significantly. The predominant RNA species synthesized by SS envelope-associated nucleoids had a sedimentation coefficient of the same order as rRNA and its precursors, with an additional low concentration of a 3·2S species. In contrast, the predominant species obtained from FS nucleoids had a sedimentation coefficient of 3·2S with no definite peaks in the 16–40S region. The nature of this low molecular weight RNA is unknown. It may be tRNA (normally 4S) or the product of endonucleolytic degradation.

FS and SS envelope-associated nucleoids contained mRNA molecules that were translatable in a cell-free synthesizing system derived from *E. coli*. To detect exogenous nucleoid mRNA translation, it was necessary to convert *E. coli* S30 extract into an mRNA-dependent translation system by destroying endogenous *E. coli* mRNA. This was accomplished by using micrococcal nuclease in the same way as described by Pelham &

Jackson (1976) for the conversion of reticulocyte lysates into an mRNA-dependent translation system. Treatment with nuclease did not alter the properties of the *E. coli*-derived protein-synthesizing machinery as was demonstrated by correct translation of coliphage MS2, *E. coli* B RNA and *B. licheniformis* A5 RNA.

Protein synthesis directed by *C. crescentus* nucleoids appeared to reflect the amount and composition of mRNA species present in the nucleoids at the time of isolation. However, the fidelity of nucleoid mRNA translation remains to be established by immunoprecipitation or enzyme activity tests on individual translation products. Nevertheless, it might be predicted that nucleoids isolated from synchronized *C. crescentus* cells could be a most useful source of stable stage-specific mRNA species.

More than twice as much protein was synthesized *in vitro* by the FS nucleoids than by SS nucleoids, which could reflect the different amounts of mRNA present in each type of nucleoid at the time of isolation. A sequential *in vitro* transcription/*in vitro* translation system was developed to detect any protein synthesis by *in vitro*-synthesized mRNA. Preincubation of isolated nucleoids in an *in vitro* transcription mixture for 40 min brought about a dramatic increase in the amount of [³⁵S]methionine incorporated into protein when the nucleoids were subsequently used as a source of mRNA. In the case of the FS nucleoids the increase was more than 10-fold, while for the SS nucleoids it was about 2-fold. These results can most easily be explained if *in vitro* mRNA synthesis has occurred during the 40 min preincubation, although other explanations involving effects on the control of protein synthesis have not been ruled out. If the FS nucleoid has a high intrinsic capacity to synthesize mRNA in the *in vitro* transcription assay, this might well explain the high background of RNA species seen between 5S and 23S which obscures the ribosomal RNA peaks. It remains to be established whether these species are due to *in vitro*-synthesized mRNA.

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