Myxococcus xanthus, a myxobacterium, contains a peculiar branched RNA-linked DNA called msDNA. Reverse transcriptase has been shown to be required for the production of msDNA. Existence of proteins that bind to one of the two msDNAs in M. xanthus, msDNA-Mx162, was examined by gel retardation assays. Total cell-free extract yielded two distinct retarded bands. Both bands were sensitive to treatment with proteinase K, indicating that there is a protein(s) that is able to bind to msDNA. Further, the formation of the bands was inhibited by the addition of nonradioactive msDNA but not by a large excess of poly(dA) in the presence of a 5000-fold excess of poly(dI·dC)·poly(dI·dC). In vivo footprinting using dimethyl sulfate revealed that the deoxynucleoside stretch from 60 to 161 is protected. When a M. xanthus cell lysate was centrifuged in a 16-30% glycerol gradient, msDNA was found to sediment in two peaks: a major peak corresponding in size to 14 S, and a minor one at 5 S. These results indicate that msDNA-Mx162 exists as a complex with specific proteins in the cell.

Myxococcus xanthus, a myxobacterium, contains proteins capable of undergoing a complex life cycle including cellular aggregation, sporulation, and fruiting body formation upon starvation (see review in Ref. 1). This organism has been thoroughly investigated as a model system for differentiation. Apart from the chromosomal DNA, M. xanthus contains two satellite DNA-RNA complexes (2, 3) whose functions are unknown. Both DNA and RNA present in these complexes are single-stranded, and DNA is branched out from the RNA molecule. One of them, designated as multicopy single-stranded DNA or msDNA (msDNA-Mx162) which occurs at a level of 500-700 copies per genome (2) is 162 bases long, and its 5' end is linked to a 77-base-long RNA at the 20th rG by a 2',5'-phosphodiester linkage. The gene encoding msDNA-Mx162 has been cloned and characterized (2). The second satellite DNA-RNA molecule, designated mrDNA (msDNA-Mx65), consists of a 65-base-long single-stranded DNA covalently linked to a 49-base-long branched RNA by a 2',5'-phosphodiester linkage (3). msDNAs have been shown to be widely distributed among various species of myxobacteria (4). A satellite DNA-RNA complex, msDNA-Sa163, occurring in Stigmatella aurantiaca has also been characterized (5, 6).

In addition to myxobacteria, msDNAs have also been recently discovered in Escherichia coli B and a clinical isolate of E. coli. Although there are no sequence homologies among msDNAs (except for the homology between msDNA-Mx162 and msDNA-Sa163), all of them share a few unique features including the 2',5'-phosphodiester linkage between the RNA and DNA components, extensive secondary structures in the RNA and DNA molecules, and DNA-RNA hybrids at their 3' ends. Earlier, we predicted that reverse transcriptase is required for msDNA biosynthesis (7). Recently, it has been shown that M. xanthus (8, 9) as well as E. coli B (10) and a clinical isolate of E. coli (11) contain reverse transcriptases, which are essential for the production of msDNA.

In this report, we attempted to elucidate whether msDNA-Mx162 is associated with other macromolecular factors within the cell. Gel retardation experiments using total cell-free extract and 32P-labeled msDNA-Mx162 treated with RNase A and T1 suggest that M. xanthus contains proteins capable of specifically binding to the msDNA molecule. Centrifugation of cell lysates in glycerol density gradients indicates that msDNA-Mx162 exists as a complex with macromolecular factors which sediments at 14 S. The in vivo and in vitro footprinting analyses also reveal specific interaction between msDNA and other components.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dimethyl sulfate was purchased from Aldrich; [α-32P]dideoxyadenosine 5'-triphosphate and [γ-32P]ATP were purchased from Amer sham Corp. Dithiothreitol and T4 polynucleotide kinase were purchased from Boehringer Mannheim; EtBr was from Calbiochem. Terminal deoxynucleotidyltransferase was purchased from International Biotechnologies, Inc. The dye reagent for Bradford's protein assay was purchased from Bio-Rad. Poly(dA) (single-stranded, 632 bases long) and poly(dI·dC)·poly(dI·dC) (double-stranded, sodium salt) were from Pharmacia LKB Biotechnology Inc. Alcohol dehydrogenase, bovine serum albumin, Brij-58, myoglobin, piperidine, proteinase K, and thyroglobulin were purchased from Sigma. All other reagents were of analytical grade.

**Methods**

**Bacteria and Growth Conditions—** M. xanthus strain DZ1 (12) was grown vegetatively in Casitone-yeast extract medium (13) at 30 °C. Culture turbidity was monitored with a Klett-Summerson colorimeter using a red filter.

**Preparation of Cell-free Extract—** Cell-free extracts for binding studies with 32P-labeled msDNA were prepared as follows. Cells grown to a density of 30-40 Klett units were harvested by centrifugation at 8,000 rpm for 10 min in an SS-34 rotor (Sorvall RC-5B Refrigerated Superspeed Centrifuge). Pelleted cells from a 50-ml culture were...
washed with 10 ml of ice-cold buffer containing 50 mM HEPES (pH 7.5), 5 mM KCl, and 1 mM MgCl₂. Washed cells were resuspended in 700 μl of the above buffer and stored at -70 °C until use. When needed, frozen cells were thawed in an ice water bath, and to each tube containing cells from a 50-ml culture 40 μl of 1 M KCl, 6 μl of 0.1 mM dithiothreitol, and 3.2 μl of 20 mM EDTA were added, and then the cells were disrupted on ice by sonication (Hercules Ultrasonics, Inc., model W-220F) at the power setting twice for 30 s with 1-min cooling intervals. The disrupted cells were spun at 108,000 × g for 25 min in an ultracentrifuge (Beckman TL-100), and the supernatant was aliquoted and stored at -70 °C until use.

**Determination of Protein Concentration**—Protein concentrations in the extract were determined by Bradford's dye-binding assay (14). Bovine serum albumin was used as the protein standard.

**Isolation of msDNA and Labeling at the 5' and 3' Ends**—msDNA from *M. xanthus* DZF1, which is devoid of most of the RNA portion because of treatment with RNase A and T₁, was prepared as described previously (2). The gel-purified DNA was recovered by electroelution and used for the end-labeling reactions. This DNA contains a ribo-trinucleotide (5' AGC 3') at its 5' end by a 2',5'-phosphodiester linkage to the RG and was labeled at the 5'-α of T₄ polynucleotide kinase. A typical kinase reaction contained 0.5 pmol of msDNA (27 ng), 50 μl of [1-³²P]ATP (specific activity >5000 Ci/mM) and 0.5 μl of T₄ polynucleotide kinase in a total volume of 10 μl in a buffer containing 66 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 10 mM β-mercaptoethanol. The reaction mixture was incubated at 37 °C for 30 min, and the labeled msDNA was separated from the unincorporated label by gel filtration on a Sephadex G-50 column as described above.

**Gel Retardation Assay**—Gel retardation assays were carried out on 4% polyacrylamide gels (acrylamide:bisacrylamide, 39:1) in TBE buffer (12) in the presence of sodium dodecyl sulfate. The S value of the msDNA was determined by layering five steps (16, 19.5, 23, 26.5, and 30%) each of a 2.1-ml volume in buffer A containing 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl₂, and 0.5 mM dithiothreitol. The gradients were stored overnight in order to allow the steps to blend and create a more continuous gradient.

Cells from *M. xanthus* DZF1 culture grown to stationary phase (200 Klett units) were collected by centrifugation and resuspended in 25% sucrose in a buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA in a 50-100-fold concentration, and 0.5-ml samples were incubated at 37 °C for 1 h. The samples were layered into an ice water bath, and 5 μl of a 5% Dri-fal solution (in 0.1 M Tris-HCl (pH 8.0)) was added to each tube. The samples were left on ice for 5 min, vortexed thoroughly, and centrifuged in an Eppendorf centrifuge at 4 °C for 5 min. The supernatant was diluted with an equal volume of buffer A containing 4 mg each of myoglobin (2 S) and bovine serum albumin (6.6 S), and the solution was used as markers for S value, and 0.7 ml was applied on top of a glycerol gradient and ultracentrifuged in an SW-41 swinging bucket rotor (Beckman model L8-70 M) at 35,000 rpm, 4 °C, for 14 h. Gradients were fractionated into 0.7-ml fractions and analyzed for the distribution of msDNA and the marker proteins.

**RESULTS**

**msDNA-binding Activity in the *M. xanthus* Cell-free Extract**—When the binding of a [³²P]-labeled msDNA probe to *M. xanthus* cell-free extracts was analyzed by gel retardation assay (Fig. 1), several retarded bands could be seen. Two bands designated as I and II were prominent. At the lowest protein concentration tested, namely 0.5 μg (lane 3), only band I could be seen, whereas at the highest concentration tested (32 μg, lane 9), only band II is seen. As the protein concentration increases, not only bands I and II but also a fast migrating band were observed. The new band is considered to be due to the degraded product(s) of msDNA by the nuclease(s) present in the extract.

**In Vivo Footprinting**—In vivo footprinting was performed by the modification of the methods described (15, 16). To a 100-ml culture of *M. xanthus* cells grown to mid-log phase (100-120 Klett units) in Casitone-yeast extract medium, 100 μl of dimethyl sulfate was added at 25 °C (lane 1). After 30 s, 1 ml of 20 mM EDTA was added to neutralize the excess dimethyl sulfate and the reaction stopped by adding 50 μl of dimethyl sulfate "stop" solution (1.5 M sodium acetate (pH 7.0), 1 mM β-mercaptoethanol, and 200 μg/ml tRNA) and vortexing. The reaction mixtures were extracted with phenol and the aqueous layer further extracted with ether. The msDNA was precipitated with ethanol, dissolved in 0.1 ml of water, and reprecipitated by adding 1 ml of butanol. The dry precipitate was dissolved in 20 μl of 2 M Tris-HCl (pH 8.0) buffer and treated with RNases (RNase A and T₁). The msDNA was purified by electrophoresis on a 6% polyacrylamide gel and recovered by electroelution, labeled at the 3' end, cleaved using piperidine, and electrophoresed on sequencing gels.

**Density Gradient Centrifugation**—Glycerol density gradient (16-30%) centrifugation was performed as described (18). The gradients were formed by layering five steps (16, 19.5, 23, 26.5, and 30%) each of a 2.1-ml volume in buffer A containing 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl₂, and 0.5 mM dithiothreitol. The gradients were stored overnight in order to allow the steps to blend and create a more continuous gradient.

**In Vivo Footprinting**—In vivo footprinting was performed by the modification of the methods described (15, 16). To a 100-ml culture of *M. xanthus* cells grown to mid-log phase (100-120 Klett units) in Casitone-yeast extract medium, 100 μl of dimethyl sulfate was added at 25 °C (lane 1). After 30 s, 1 ml of 20 mM EDTA was added to neutralize the excess dimethyl sulfate and the reaction stopped by adding 50 μl of dimethyl sulfate "stop" solution (1.5 M sodium acetate (pH 7.0), 1 mM β-mercaptoethanol, and 200 μg/ml tRNA) and vortexing. The reaction mixtures were extracted with phenol and the aqueous layer further extracted with ether. The msDNA was precipitated with ethanol, dissolved in 0.1 ml of water, and reprecipitated by adding 1 ml of butanol. The dry precipitate was dissolved in 20 μl of 2 M Tris-HCl (pH 8.0) buffer and treated with RNases (RNase A and T₁). The msDNA was purified by electrophoresis on a 6% polyacrylamide gel and recovered by electroelution, labeled at the 3' end, cleaved using piperidine, and electrophoresed on sequencing gels.
msDNA Complex of M. xanthus

13667

n-I-rnsDNA-Degraded rnsDNA

FIG. 1. Titration of extract proteins with the \textsuperscript{32}P-labeled msDNA probe. Increasing amounts of the total cell-free extract (0.5, 1, 2, 4, 8, 16, and 32 \textmu g of protein in lanes 5, 4, 5, 6, 7, 8, and 9, respectively) were mixed with 0.1 ng (1.82 fmol) of msDNA labeled at its 5' end with \textsuperscript{32}P in a volume of 12 \textmu l, incubated at 25 \degree C for 30 min, and electrophoresed. Lane 2 presents results for 32 \textmu g of bovine serum albumin similarly processed. Lane 1 contains a sample to which no protein was added to indicate the location of free labeled msDNA. The signs + or - indicate the presence or the absence of extract proteins.

FIG. 2. Effect of increasing KCl concentration on the binding of \textsuperscript{32}P-labeled msDNA to the cell extract. The extract (10 \textmu g of protein) was incubated with 0.1 ng of the probe for 30 min in the presence of different concentrations of KCl (0.1, 0.2, 0.4, 0.8, 1.2, and 2 M KCl in lanes 2, 3, 4, 5, 6, and 7 respectively) and electrophoresed. Lane 1 presents results for msDNA at 0.1 M KCl without added extract. The signs + and - indicate the presence and the absence of the extract proteins, respectively.

Effects of Proteinase K and Ethidium Bromide—In order to examine whether bands I and II are the result of binding of proteins to the msDNA probe, the extract was pretreated with proteinase K and then used for the binding assay (Fig. 3). As shown in lanes 4–7 in Fig. 3, both bands I and II disappeared when the extract was treated with proteinase K, clearly demonstrating that the formation of bands I and II is due to the association of proteins with msDNA.

The effect of ethidium bromide (EtBr), an intercalating agent, was also tested (Fig. 4). Both bands I and II disappeared at 0.2 mM (lane 4), but their sensitivities toward increasing EtBr concentrations were different. The intensity of band II decreased with increasing concentrations of EtBr, whereas that of band I increased at 0.05 mM (lane 2) and at 0.1 mM (lane 3) EtBr concentrations and disappeared at 0.2 mM EtBr (lane 4). EtBr was able to abolish the appearance of bands I and II at 0.2 mM concentration whether added prior to forming the DNA-protein complex or after forming the complex.

FIG. 3. Proteinase K susceptibility of the factors involved in binding to the msDNA probe. The extract (6 \textmu g of protein) was pretreated with proteinase K (1.5 \textmu g) at 25 \degree C for 0 (lane 4), 15 (lane 5), 30 (lane 6), and 60 min (lane 7) and then incubated with 0.1 ng of the probe at 25 \degree C for 30 min as described and assayed. Lane 3 presents the result for the binding of msDNA to the extract without proteinase K treatment. Lane 2 presents the results in which msDNA was treated with proteinase K, and lane 1 is msDNA without extract and proteinase K. The proteinase K digestion and the binding reactions were carried out at 400 mM KCl.

FIG. 4. Effect of ethidium bromide on the binding of the extract protein to msDNA. The \textsuperscript{32}P-labeled msDNA probe (0.1 ng) was incubated with 0.05 (lane 2), 0.1 (lane 3), and 0.2 mM (lane 4) concentrations of EtBr for 10 min and mixed with the extract (5 \textmu g of proteins). The mixture was then incubated for an additional 30 min at 25 \degree C and assayed as described in Fig. 1. Lane 1 presents the result for the reaction carried out without EtBr. The binding reactions were carried out at 400 mM KCl.
It is known that the primary binding of EtBr to DNA occurs by a process of intercalation between adjacent base pairs, whereas secondary binding occurs by a “stacking” mechanism (19). It is possible that EtBr at lower concentrations (probably up to 0.1 mM) intercalated into msDNA, which resulted in a change in the conformation of msDNA. This conformational change is unfavorable for the formation of band II but favorable for the formation of band I.

Specificities of the Proteins That Bind to msDNA—The specificities of the proteins that bind to the msDNA probe were examined by competition experiments with unlabeled msDNA and poly(dA). Since band I preferably appears in 100 mM KC1 and band II in 400 mM KC1, the competition experiments were carried at both 100 mM KC1 (Fig. 5A) and 400 mM KC1 (Fig. 5B). It can be seen from Fig. 5A that band I is almost abolished by a 50-fold concentration of unlabeled msDNA (lane 8), whereas even a 100-fold (the weight of the msDNA probe used) concentration of poly(dA) could not abolish band I completely (lane 16). However, at higher concentrations, there seems to be a little inhibition of the binding. In a similar experiment at 400 mM KC1 (Fig. 5B), both bands I and II were almost completely abolished by a 50–100-fold concentration of unlabeled msDNA (lanes 8 and 9, respectively), whereas the binding was not affected by a 100-fold concentration of poly(dA) (lane 16). It should be noted that the binding is carried out in the presence of a 5000-fold excess of poly(dI-dC)poly(dI-dC), a double-stranded DNA polymer. From these results, one could conclude that both bands I and II are the result of binding of specific proteins to msDNA. It is also evident from lane 2 of Fig. 5, A and B that even as low as 0.25 μg of the protein extract could produce intense bands I and II, indicating that these specific binding proteins are abundant in the extract.

In Vivo Footprinting—Since the gel retardation experiments indicated the presence of proteins that bind to msDNA specifically, we next performed in vivo footprinting experiments to examine which parts of msDNA are associated with proteins in the cell. For this purpose, exponentially growing cells were first treated with dimethyl sulfate. The msDNA was then extracted and subjected to Maxam and Gilbert sequencing reactions as described under “Experimental Procedures.” Fig. 6, A and B, shows the sequencing gel patterns on an 8% acrylamide gel for different time periods, and Fig. 6C shows the sequencing gel pattern on an 18% gel. The tightly protected areas are shown within brackets (Fig. 6). These results are superimposed on the structure of msDNA-Mx162 in Fig. 7. One can observe that the DNA sequence covering the bases 60–161 (shaded box in Fig. 7) was protected from the action of dimethyl sulfate. Cleavage of G residues in the region covering bases 1–59 (Fig. 6A) is poor, probably due to secondary structure. Hence, we were unable to determine whether the DNA sequence covering bases 1–59 is protected. This could be solved if 5′ end-labeled msDNA is used instead of the 3′ end-labeled msDNA, but since there is an rA at the 5′ end, the label is hydrolyzed during piperidine treatment if 5′ end-labeled msDNA is used (2).

Density Gradient Centrifugation of the Complex—Since in vivo footprinting experiments indicated that msDNA is associated with proteins, we further attempted to characterize the protein-msDNA complex by centrifugation of total cell lysates in glycerol gradients. As shown in Fig. 8, msDNA was found as the major peak at fraction 9 (peak I). From the relative position of this peak to internal standards, the S value of the complex was calculated to be approximately 14. There is another minor peak at fraction 4 (peak II) which corresponds approximately to 5 S. Free msDNA (extracted from cells, treated with ribonuclease, and labeled at the 5′ end) under these conditions sediments in fraction 2 (data not shown) along with myoglobin (2 S). This result provides additional evidence to show that msDNA is associated with proteins and exists as a complex within the cell.

In Vitro Footprinting—In vitro footprinting was also performed on the 14 S msDNA complex from fraction 9 of the glycerol gradients. Fig. 9, A and B, presents sequencing gel patterns on 8% gels run for different time periods, and Fig. 9C shows the sequence pattern on an 18% gel. The DNA sequences from 63 to 85 are partially protected, and those from 86 to 118 and 122 to 150 are protected (bracketed regions in Fig. 9). The sequences from 11 to 19 and 152 to 156 appear to have enhanced cleavages.

**DISCUSSION**

When the 32P-labeled msDNA probe prepared after treatment with ribonuclease A and T1 was mixed with the cell-
Fig. 6. In vivo footprinting of the msDNA using dimethyl sulfate. In vivo footprinting was carried out as described under “Experimental Procedures.” Lane 1 represents the results for the G-specific reaction carried out on msDNA purified from M. xanthus cells not treated with dimethyl sulfate to serve as reference. This DNA was treated with 1 µl of dimethyl sulfate after 3' end labeling and subsequently cleaved with piperidine and similarly processed. (A) 8% sequencing gel electrophoresed for 3 h. (B) 8% sequencing gel electrophoresed for 1.75 h. (C) 18% sequencing gel electrophoresed for 2 h. The numbers indicated in the autoradiograph correspond to the nucleotide sequence position on the msDNA Mx162 molecule as shown in Fig. 7.

free extract, it associated with proteins in the extract to form two distinct bands, I and II. Proteins binding to bands I and II appear to be different because of the following facts. First, the protein(s) involved in the formation of band II is labile even during storage at -70 °C. When the cell extract was stored at -70 °C for 2–3 months, the extract loses the ability to form band II, whereas band I is still formed. Second, freezing and thawing severely affected the intensity of band II, whereas band I is not affected. Third, band II was not observed in extracts from cells in the late log or stationary phases, but band I is still formed (data not shown). During these phases, band II protein(s) may disappear because of its low production in the late log phase or because of instability. Another possibility is that during the late log phase, the protein(s) may be already completely saturated with msDNA within the cell and hence not available for binding to exogenously added msDNA.

The effect of various factors on the formation of bands I and II could be explained by assuming that band I results from the binding of a set of protein(s) to msDNA, to which

Fig. 7. Protected regions of msDNA-Mx162 in in vivo footprinting. The protected DNA sequences are shown by the shaded box on the basis of the results in Fig. 6.
in turn another set of protein(s) binds, leading to the formation of band II. Alternatively, it is possible that the msDNA probe used exists in two interconvertible conformations, I and II, and there are specific proteins capable of binding to each of them in the M. xanthus extract. In the binding buffer containing 100 mM KCl, probably most of the probe exists in form I, and at high KCl concentrations, the equilibrium shifts in favor of form II. Similarly, other salts such as NaCl, NH4Cl, MgCl2, and EDTA are considered to affect the conformation of msDNA. It is also interesting to note that when increasing amounts of the extract are added to a fixed amount of [32P] msDNA, band I is prominent at low concentrations, whereas band II becomes prominent at higher concentrations. This indicates that even at low KCl concentrations, band II can be formed if there are enough band II proteins.

The results of in vivo footprinting performed on exponentially growing cells show that a substantial part of the msDNA molecule (from base 60 to 161, see Fig. 7) is not accessible for chemical modification by dimethyl sulfate, indicating that this region is protected by other factors, most likely proteins. The results of in vitro footprinting performed on a fraction corresponding to the 14 S complex in glycerol gradients were similar to those of in vivo footprinting except that (a) there was enhanced cleavage of bases 11-14, 18, and 19 (see Fig. 7); (b) the region from base 152 to 156, which is tightly protected under in vivo conditions, had enhanced cleavage; and (c) the sequence from base 63 to 85 was partially protected. The difference in the patterns of protection against dimethyl sulfate under the in vivo and in vitro conditions could be due to differences in the environment of the complex (pH, ionic strength), or some factors may be dissociated from the complex during the course of isolation of the 14 S complex.

Glycerol gradients have been used to isolate 5 S RNA particles from nuclear extracts of Xenopus oocytes (18) and 275 S virus-like particles from Saccharomyces cerevisiae containing a Ty element (20). Hence, we chose to use glycerol gradients to isolate the msDNA complex. In the glycerol gradients, msDNA is found distributed in two peaks corresponding to 14 and 5 S. At present, it is not clear how these two complexes are related. About 75% of the msDNA was found to exist in the complexed form (data not shown). Further purification and characterization of protein components associated with these complexes will provide a clue to the structure of these complexes.

We do not know at present whether the RNA moiety of msDNA-Mx162 is also bound to proteins. The other msDNA, namely msDNA-Mx65 (3), sedimented in fractions corresponding to about 8 S in the glycerol gradients, suggesting that it also exists as a complex with proteins. Currently, we are engaged in the purification of these complexes for identification and characterization of the individual factors involved. The purification of specific proteins bound to
msDNA-Mx162 is also in progress.

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