Structure of an Inducibly Methylatable Nucleotide Sequence in 23S Ribosomal Ribonucleic Acid from Erythromycin-Resistant *Staphylococcus aureus*†

Ching-juh Lai, James E. Dahlberg‡ and Bernard Weisblum*

ABSTRACT: The appearance of N^6,N^6-dimethyladenine (m_2^6A) residues in 23S rRNA is associated in certain resistant strains of *Staphylococcus aureus* with erythromycin-induced resistance to macrolides, lincosamides, and streptogramin B-type antibiotics. Fingerprint analysis of a T_1 ribonuclease (RNase) digest of 23S rRNA from uninduced (*i.e.*, sensitive) cells reveals a total of seven methyl-containing oligonucleotides. One additional methyl-containing oligonucleotide is found if the digest is prepared from induced or constitutively resistant cells. This oligonucleotide has the following properties: (1) It is a tetranucleotide. (2) It is resistant to pancreatic RNase. (3) It is the sole oligonucleotide that contains m_2^6A and m_2^6A is the only methyl-labeled component in this oligonucleotide. (4) The ratio of m_2^6A to adenine is 1:2. From further studies with labeled adenine, the ratio of m_2^6A to adenine in 23S rRNA from induced cells is 0.35%, which corresponds to two to three adenine residues per 23S rRNA. Similar analysis of constitutively resistant cells yields a ratio of approximately 1–2 m_2^6A residues/23S rRNA. The additional oligonucleotide is derived by methylation of the sequence ApApApG, and from the observed stoichiometry, there may be two or more copies of this methylatable sequence in 23S rRNA. The 3′ and 5′ end groups of 23S rRNA obtained following digestion with T_1 RNase have been separated and identified. They are the same in resistant and sensitive cells, and their mobilities on electrophoresis differ from that of the methyl-containing fragment that appears on induction. Therefore, the new methylated oligonucleotide originates in a part of 23S rRNA that is represented in the mature ribosome of the sensitive cell and is not derived from a portion of a putative precursor of 23S rRNA that might remain uncleaved owing to induction. These results also demonstrate that the erythromycin-resistant phenotypes of induced and constitutively resistant organisms, at the cellular level, correspond to qualitatively similar ribosome phenotypes at both the structural and functional levels, as well.

Altered ribosome structure and function have been found in strains of *Staphylococcus aureus* which display erythromycin-inducible resistance (Chabbert, 1956; Saito *et al.*, 1971; Lai and Weisblum, 1971). In these strains, erythromycin induces resistance to three classes of antibiotics that act on the 50S ribosome subunit—the macrolides, lincosamides, and streptogramin B-type antibiotics (Weisblum and Demohn, 1969). Ribosomes from induced or constitutively resistant cells show reduced affinity for erythromycin, and the induction of a putative erythromycinase has been excluded (Weisblum *et al.*, 1971). We have recently shown that ribosomes from induced or constitutively resistant cells contain a methylated base, N^6,N^6-dimethyladenine, in 23S rRNA, that is absent from the rRNA of sensitive or uninduced cells (Lai and Weisblum, 1971). Studies from other laboratories (Saito *et al.*, 1971) as well as our own have failed to detect ribosomal protein alterations in these resistant strains. We present below a more detailed account of the changes in methylation of the 23S rRNA obtained from resistant cells.

Materials and Methods

Bacterial strains used in this study were *S. aureus* 1206, a standard erythromycin-inducible strain, and a constitutively resistant, spontaneous mutant derived from 1206. Some of their properties, including antibiotic resistance patterns, have been described (Weisblum *et al.*, 1971).

Bacterial cells were grown in an enriched broth medium which contained (in grams per liter), yeast extract, 5; bactopeptone, 5; glucose, 2; and K_2HPO_4, 1. For purposes of labeling RNA, the medium was diluted 5-fold with water and supplemented with either [^{14}C-*methyl*]methionine, specific activity 50 Ci/mol, 0.2 μCi/ml; or [8-^{14}C]adenine, specific activity 58 Ci/mol, 1 μCi/ml; or [8-^{3}H]adenine, specific activity 26,000 Ci/mol 1 μCi/ml; or $^{32}P_i$, carrier free, 10 μCi/ml, as indicated. For induction, 10^{-7} M erythromycin was used. rRNA was prepared as described previously (Lai and Weisblum, 1971). For fingerprint analysis, digestion and fractionation were performed according to the methods described by Sanger *et al.* (1965). The 3′ and 5′ end groups were obtained by the technique of Dahlberg (1968).

Results

m_2^6A has been found in 23S rRNA of *S. aureus* if inducible cells are grown in the presence of 10^{-7} M erythromycin; this base can also be demonstrated in constitutively resistant cells grown in the absence of erythromycin (Lai and Weisblum, 1971). Therefore, we attempted to identify oligonucleotides in which m_2^6A might be localized.

Three preparations of ^{14}C-methyl-labeled 23S rRNA were made by incubation of growing cultures with [^{14}C-*methyl*]methionine. Induced, uninduced and constitutively resistant cells were used. 23S rRNA prepared from the cells of each culture was digested with T_1 RNase plus *Escherichia*

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‡ Department of Physiological Chemistry.

TABLE I: Quantitation of Dimethyladenine.

Sample	Label	Cell Type	Adenine (cpm)	m_2^6A (cpm)	m_2^6A/Adenine (%)	mol of m_2^6A/mol of Sample
23S rRNA	[8-^{3}H]Adenine[a]	Uninduced control	162,000	40	<0.01	
		Induced	102,000	367	0.35	2.8
		Constitutive	577,000	804	0.14	1.2
Oligonucleotide	[8-^{14}C]Adenine[b]	Uninduced control				
		Induced	175	80	46.0	1.0

[a] [8-^{3}H]Adenine-labeled 23S rRNA was depurinated and fractionated into its adenine and dimethyladenine constituents. The relative amount of radioactivity in adenine and dimethyladenine was determined. [b] [8-^{14}C]Adenine-labeled 23S rRNA was enzymatically digested and fractionated by the fingerprint method as described. The oligonucleotide that is only present in resistant cells was eluted and depurinated and the resultant digest was fractionated by paper chromatography (isopropyl alcohol–concentrated ammonia–water, 70:1:29; Whatman No. 3MM, descending, 18 hr). The fractions containing adenine and dimethyladenine were located and eluted and the relative amount of radioactivity in adenine and dimethyladenine was determined by counting the fractions in a gas-flow counter.

coli alkaline phosphatase. The resultant digests were fractionated according to the method of Sanger *et al.* (1965) and autoradiograms were prepared. The results are summarized in a schematic fashion in Figure 1.

The 23S rRNA from uninduced cells, yields seven methyl-containing oligonucleotides. Except for the presence of a single additional component, the patterns of methylation of 23S rRNA from induced and constitutively resistant cells are indistinguishable from this control. The new oligonucleotide appears to have the same mobility in both resistant strains.

The new oligonucleotide was further purified and characterized by chromatography on DEAE-Sephadex according to the method of Tomlinson and Tenner (1963). Under these conditions the new component fractionated as a single radioactive peak that had the same mobility as the tetranucleotide fraction of a T_1 RNase digest of yeast rRNA. Moreover, incubation with pancreatic RNase did not alter the electrophoretic mobility of the oligonucleotide (7% formic acid, or pyridine–acetate, pH 3.5; DEAE paper). These results suggest that the new oligonucleotide was obtained from the methylation of the sequence ApApApG.

All eight methyl-labeled oligonucleotides from the fingerprints shown in Figure 1 were eluted from the paper and hydrolyzed with acid. The acid hydrolysate was fractionated by paper chromatography. m_2^6A was the only radioactive component in the hydrolysate of the new oligonucleotide; only the new methyl-labeled oligonucleotide contained m_2^6A. These results suggest that the new oligonucleotide does not arise from a sequence that is normally methylated in sensitive cells. Moreover, these findings rule out the possibility of other forms of methylation as a possible cause of resistance to pancreatic RNase.

In order to determine the relative amounts of m_2^6A and adenine in 23S rRNA and in the new oligonucleotide, cells were incubated with labeled adenine. 23S rRNA labeled with [^{3}H]adenine was purified and acid hydrolyzed. The fractions containing adenine plus methylated adenines (obtained by column chromatography on Dowex 50) were further fractionated by paper chromatography, and the relative amount of radioactivity in adenine and m_2^6A was determined. The results shown in Table I indicate the presence of 2–3 m_2^6A residues/23S rRNA for the induced, and 1–2 m_2^6A residues/23S rRNA for the constitutively resistant cells.

In order to determine the ratio of m_2^6A to adenine in the new oligonucleotide, 23S rRNA labeled with [^{14}C]adenine was used. In this case it was found useful to omit phosphatase from the digestion in order to obtain the methylated oligonucleotide pure of other labeled oligonucleotides. It should be pointed out that the unmethylated counterpart of this methylated oligonucleotide has a different mobility. This allows us to study the purified methylated adenine-labeled oligonucleotide for a determination of the mole fraction of m_2^6A. Following enzymatic digestion and fractionation, the new methylated oligonucleotide was hydrolyzed with acid, and the resulting adenine and dimethyladenine were purified. The relative amount of radioactivity in the two fractions was 2:1, respectively. This suggests that, of three adenines, only

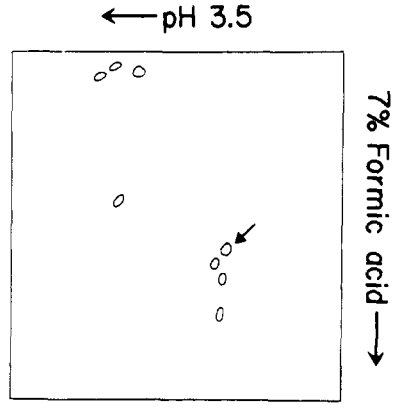

FIGURE 1: Fingerprint analysis of 23S rRNA labeled with [^{14}C-*methyl*]methionine. [^{14}C]Methyl-labeled 23S rRNA was prepared as described in Materials and Methods. The RNA (100 μg) was digested with 5 μg of T_1 RNase (Sankyo) plus 10 μg of bacterial alkaline phosphatase (Worthington BAP-F) in a total volume of 10 μl of 0.01 M Tris-HCl (pH 8.0) for 30 min at 37°. The resulting oligonucleotides were fractionated by the technique of Sanger *et al.* (1965) and located by autoradiography. Arrow indicates position of dimethyladenine-containing oligonucleotide that is present only in RNA from induced or constitutively resistant cells.

one is methylated. It should be noted that this figure represents *at least* one residue rather than an average value; an *average* value of 1 would be arithmetically impossible since the methylated oligonucleotide was free of its unmethylated precursor, ApApApG. Taking into consideration that 2–3 m_2^6A/23S rRNA were found, we conclude that the methylatable sequence may be duplicated.

The ApApApG sequences that are dimethylated could come from one of two sources. They could be part of the active 23S rRNA found in sensitive cells or they could be part of a precursor of 23S rRNA (Dahlberg and Peacock, 1971) that is enzymatically removed in sensitive (but not resistant) cells during ribosome maturation. The results described do not discriminate between these possibilities. If the latter mechanism applied, we might expect to see differences in their relative sizes and in the oligonucleotides at the 3′ and 5′ ends of 23S rRNA from sensitive and resistant cells. To test this possibility, 23S rRNAs from sensitive and resistant cells were fractionated by electrophoresis in agarose–polyacrylamide; no differences were seen in their relative mobilities.

The 3′ and 5′ ends were purified by the "phosphatase diagonal" method (Dahlberg, 1968); the 3′- and 5′-terminal oligonucleotides from resistant and sensitive cells were the same in both types of RNA. Subject to the limitations of negative data, these results suggest that the methylatable oligonucleotide is derived from a part of the *mature* 23S rRNA of sensitive cells.

Discussion

The results obtained in this study indicate that a single type of change in 23S rRNA occurs upon induction, and as far as we can ascertain, it involves only the appearance of m_2^6A. Whereas the column method employed previously to demonstrate m_2^6A does not ensure the recovery of all oligonucleotides that might be of interest, fingerprint analysis used here allows us to examine *all* methylated oligonucleotides. Under these conditions the dimethylation of adenine remains the only detectible change in RNA. Moreover, there is no apparent qualitative difference between the fingerprints obtained from induced and constitutively resistant cells. These observations support our previous conclusion that constitutively resistant cells express the same biochemical mechanism of resistance as do induced cells. Further indirect support comes from a genetic analysis of similar erythromycin-inducible and constitutively resistant strains of *S. aureus* in which Bronson and Pattee (1972) have concluded that only one genetic locus is involved.

A multiplicity of methylating enzyme activities, at least one of which is specific for adenine in 16S and another for 23S rRNA, is suggested by the observation that m_2^6A is present in 16S rRNA from induced, uninduced, and constitutively resistant cells in similar amounts. We could not detect any methylated oligonucleotide from 16S rRNA with the same mobility as the new oligonucleotide from 23S. There are likely to be additional adenine methylating enzymes specific for tRNAs as well as for DNA. It is our working hypothesis that the new methylating activity arises from enzyme synthesis as a consequence of derepression by erythromycin; however, other models involving positive control or modification of preexisting methylating enzymes cannot yet be ruled out.

A multiplicity of methylatable A-A-A-G sequences in 23S rRNA is suggested by the stoichiometry of methylated adenine. This concept is an important one to establish in order to explain several unexpected observations made in previous studies. First, we noted earlier that while *in vitro* erythromycin binding by ribosomes from induced cells was decreased, it was not completely reduced to background as in the case of constitutively resistant cells (Weisblum *et al.*, 1971). If only one methylatable site were involved, one would expect that different degrees of methylation would affect only the plateau level of binding. Actually, induction appeared to affect mainly the slope of the curve relating complex formation with erythromycin input. Second, in studies of constitutive resistance, we obtained mutant strains that appeared to be only partially constitutive. Both these sets of observations might be clarified if there existed at least two different sites in 23S rRNA for adenine methylation and that methylation of only one site conferred partial resistance. It may be possible to test this hypothesis critically by preparing fingerprints of longer fragments from 23S rRNA in which the m_2^6A-containing oligonucleotides would be longer than the tetranucleotide described. It should be mentioned that the existence of two copies of certain methylatable oligonucleotide sequences in 23S rRNA of *E. coli* has been proposed by Fellner (1969).

Altered methylation of 16S rRNA in kasugamycin-resistant *E. coli* has been described by Helser *et al.* (1971). The detailed mechanism of resistance to this drug is different from that described here because it is the *nonmethylated* form that is resistant. This is due to the loss or inactivation of an enzyme normally present that converts adenine to m_2^6A (Helser *et al.*, 1972). Thus kasugamycin resistance in *E. coli* is due to a specific "under" methylation, whereas erythromycin resistance in *S. aureus* is due to a specific "over" methylation. In this connection, we have begun a survey of gram-positive organisms and have noted that methylated adenines are absent from the 23S rRNA of an erythromycin-sensitive strain of *Streptococcus pyogenes* (group A, hemolytic), whereas dimethyladenine can be found in a (nonisogenic) natural isolate resistant to erythromycin and lincomycin; when tested for antibiotic sensitivity, we found that this strain was resistant to vernamycin Bα but sensitive to vernamycin A. Thus the constitutive phenotype described originally for *S. aureus* may have a wider phylogenetic distribution.

A central issue in these studies is the relationship between methylation and resistance; *i.e.*, Is methylation causally related to resistance? It has recently been possible to reconstitute heterologous 50S ribosomal subunits using 23S rRNA from induced or uninduced *S. aureus* plus other components from erythromycin-sensitive *Bacillus stearothermophilus* (Lai *et al.*, 1973) and resistance in the reconstituted particles appears to be associated with the 23S rRNA from resistant cells.

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Transcription of Ribonucleic Acid by the Ribonucleic Acid Directed Deoxyribonucleic Acid Polymerase of Rous Sarcoma Virus and Deoxyribonucleic Acid Polymerase I of *Escherichia coli*†

J. M. Taylor,* A. J. Faras, H. E. Varmus, H. M. Goodman, W. E. Levinson, and J. M. Bishop

ABSTRACT: The DNA synthesized *in vitro* by the purified RNA-directed DNA polymerase of Rous sarcoma virus (RSV), using tumor virus RNA as template, contains both single- and double-stranded molecules. Both forms are small and the double-stranded form is copied primarily from particular regions of the RNA. These same properties are found to apply to the DNA transcribed from poliovirus RNA. The primer $(dT)_{12-18}$ augments the response of the enzyme to RSV RNA, heat-denatured RSV RNA, and poliovirus RNA. The size of the DNA products is still small and the portion of the RNA template represented in double-stranded DNA is decreased. In transcription from RSV RNA, the use of $(dT)_{12-18}$ produces additional initiation sites on the template although DNA is not transcribed from regions other than those previously transcribed. DNA synthesis on 70S RSV RNA template is initiated by the formation of a phosphodiester bond between a 3′-terminal adenosine moiety and deoxyadenosine. Addition of $(dT)_{12-18}$ stimulates the overall synthesis of DNA and does not interfere with the formation of the above-mentioned bond. The DNA-dependent DNA polymerase I of *Escherichia coli* does not respond to naturally occurring RNA templates. However, addition of $(dT)_{12-18}$ to poliovirus RNA and RSV RNA, which possess poly(A) sequences, permits the synthesis of DNA limited to poly(dA) and poly(dT) rather than heteropolymer.

The RNA-directed DNA polymerase of RNA tumor viruses has been purified in several laboratories (Kacian *et al.*, 1971; Duesberg *et al.*, 1971a; Hurwitz and Leis, 1972; Faras *et al.*, 1972). These preparations are entirely dependent on the addition of exogenous template and are sufficiently free of ribonuclease activity to allow the use of ribonuclease-sensitive naturally occurring RNA templates. They respond to DNA and to synthetic homopolymers (such as poly(dA·dT) and poly(A)·oligo(dT)), but display a preference for 70S oncornavirus RNA as template among the naturally occurring RNAs tested (Duesberg *et al.*, 1971a; Faras *et al.*, 1972). The DNA transcribed from 70S RNA has been generally found to possess the following properties. First, the DNA molecules are small (4–10 S) relative to the size of the oncornavirus genome (reviewed by Temin and Baltimore, 1973). Secondly, transcription into ds-DNA[1] occurs predominantly from very limited regions of the genome (Gelb *et al.*, 1971; Varmus *et al.*, 1971; Taylor *et al.*, 1972). These findings led us to investigate whether the same two properties of the DNA product apply to other situations of RNA-directed DNA synthesis. We have used the purified polymerase of Rous sarcoma virus (RSV) to study the transcription of RSV and poliovirus RNAs, with particular emphasis on the effects of an exogenously added primer molecule $(dT)_{12-18}$ (Duesberg *et al.*, 1971b). In addition, we have analyzed the nature of RNA-directed DNA synthesis by DNA polymerase I of *Escherichia coli*.

Materials and Methods

Reagents and Solutions. Deoxynucleoside triphosphates were from Calbiochem. [^{3}H]Deoxynucleoside triphosphates (5–24 Ci/mmol) were from Schwarz BioResearch, Inc. [α-^{32}P]-Nucleoside triphosphates (4–16 Ci/mmol) were from International Chemical and Nuclear Corp. $(dT)_{12-18}$ was from Collaborative Research, Inc.

† From the Departments of Microbiology and Biochemistry, University of California, San Francisco, California 94122. *Received July 3, 1972.* This investigation was supported by U. S. Public Health Service Grants AI 08864, CA 12380, CA 12705, AI 06862, and AI 00299, American Cancer Society Grant P-591, and Contract 71-2147 within the Special Virus-Cancer Program of the National Cancer Institute, the National Institutes of Health, and the Public Health Service. J. M. T. and H. E. V. acknowledge the support by Senior Dernham Fellowships (D-201 and D-164, respectively), of the American Cancer Society, California Division.

[1] Abbreviations used are: ds-DNA, double-stranded DNA; ss-DNA, single-strand DNA; RSV, Rous sarcoma virus; C_0t, concentration × time, expressed as (mol sec)/l.