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Modification of Cytidines in a Qβ Replicase Template: Analysis of Conformation and Localization of Lethal Nucleotide Substitutions†

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ABSTRACT: The solution conformation of MDV-1(+) RNA, a small RNA template replicated autocatalytically in vitro by Qβ replicase, was investigated with sodium bisulfite, a reagent that selectively converts single-stranded cytidines to uridines. The reactivity of 45 of the 76 cytidines in MDV-1(+) RNA was determined by nucleotide sequence analysis. Only 14 of these 45 cytidines were converted to uridine. Treatment of the RNA with methoxyamine, another single-strand-specific cytidine modification reagent, gave results in good agreement with the bisulfite data. The limited reactivity of MDV-1(+) RNA with these reagents indicates that it is a highly structured molecule. A secondary structure consistent with the chemical modification data is proposed. Modification of MDV-1(+) RNA by bisulfite renders it inactive as a template for RNA replication. This inactivation and the modification of the cytidines at the 3′ end of the molecule occur at very similar rates. By using a short complementary RNA "mask" to protect just these cytidines, we demonstrated that the loss of activity resulted from their modification. This implies that one or more of the cytidines in the 3′-terminal sequence is required for template activity and that changes within this sequence can have lethal consequences. The effects of modification elsewhere in the sequence are discussed.

Qβ replicase is the RNA-directed RNA polymerase that replicates the single-stranded RNA genome of coliphage Qβ [Haruna & Spiegelman, 1965; see Blumenthal & Carmichael (1979) for a review]. This enzyme displays a high degree of template specificity for Qβ RNA and its complement over cellular or other phage RNAs (Haruna & Spiegelman, 1965; Feix et al., 1968; Okada et al., 1971). Only two other types of RNAs are templates for autocatalytic replication by Qβ replicase in vitro: the "Qβ RNA variants" and the so-called "6S RNAs".[1] The Qβ variants were generated in vitro by extensive replication of Qβ RNA (Mills et al., 1967). These RNAs, which hybridize to Qβ RNA (Mills et al., 1968), are approximately 550 nucleotides long. The 6S RNAs actually range in size from 91 to 221 nucleotides. They were originally isolated from in vitro Qβ replicase reactions that did not

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[1] Qβ replicase carries out a limited polymerization on certain other templates, but they are not replicated autocatalytically [see Schaffner et al. (1977) for a summary].

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contain exogenous template RNA (Banerjee et al., 1969; Kacian et al., 1972; Mills et al., 1975; Schaffner et al., 1977). Although these RNAs show some sequence homology with Qβ RNA (Mills et al., 1973; Schaffner et al., 1977), their evolutionary relationship to the viral genome remains unclear (Banerjee et al., 1969; Sumper & Luce, 1975; Schaffner et al., 1977). MDV-1 RNA (Kacian et al., 1972), one of the 6S RNAs whose complementary strands have been purified to homogeneity (Mills et al., 1978), is the subject of the chemical modification studies reported here.

MDV-1 RNA is an excellent model template for studying the mechanism of RNA replication. It is small (each complementary strand is only 221 nucleotides long), and its complete nucleotide sequence has been determined (Mills et al., 1973). The two complementary strands, MDV-1(+) RNA and MDV-1(−) RNA, are found in synthetic reactions as single-stranded molecules (Kacian et al., 1972). Qβ replicase selectively recognizes and binds to either complement and can then use that single-stranded RNA as a template for synthesis of a complementary strand (Mills et al., 1977). Product strand synthesis is initiated at the 3′ end of the template strand, and polymerization of the product proceeds in the 5′ to 3′ direction. During chain elongation, the nascent product and its template are essentially single stranded. After the product strand is completed, the replication complex dissociates, freeing the template and product (Dobkin et al., 1979). Because the product strand may itself serve as a template, synthesis of RNA by Qβ replicase is "autocatalytic".

There are many indications that the complementary single strands of MDV-1 RNA are highly structured. The nucleotide sequence of each strand possesses many neighboring intrastrand complements that are capable of forming stable hairpin structures (Mills et al., 1973). Each strand is very resistant to single-strand-specific ribonucleases (Mills et al., 1973). And, the electrophoretic mobility of MDV-1 RNA fragments indicates the presence of many secondary structures (Kramer & Mills, 1978; Mills & Kramer, 1979).

The secondary and tertiary structures of MDV-1 RNA probably provide signals to the replicase that are required for synthesis. Since Qβ replicase does not bind to a duplex of the complementary strands, specific recognition may depend on the conformation of the single-stranded RNA (Mills et al., 1977). The formation of structures in the product strand may be required during chain elongation to prevent reassociation of the product with the template (Dobkin et al., 1979). Furthermore, the rate of chain elongation is highly variable and is controlled by the formation of secondary structures in either the product or the template strand (Mills et al., 1978). Other aspects of replication, including product chain initiation and the release of the completed product strand, are also likely to require particular structural features. We therefore performed experiments designed to study the conformation of MDV-1 RNA.

In this paper we report the results of experiments that are based on the modification of MDV-1(+) RNA by sodium bisulfite and methoxyamine, two reagents that specifically modify cytidines occurring in single-stranded regions of RNA. Sodium bisulfite catalyzes the conversion of cytidine to uridine (Shapiro et al., 1970; Hayatsu et al., 1970), a reaction which is strongly inhibited if the cytidine residue is involved in secondary or tertiary polynucleotide structure [Goddard & Schulman, 1972; Shapiro et al., 1973; Lowdon & Goddard, 1976; Bhanot & Chambers, 1977; see Hayatsu (1976) for a review]. Susceptibility to modification implies that a cytidine exists in an accessible unstacked single-stranded conformation, whereas resistance indicates involvement in an ordered structure. Similar information is obtained with methoxyamine, a reagent that selectively forms adducts with cytidines in single-stranded regions (Cashmore et al., 1971; Chang, 1973; Robertus et al., 1974; Rhodes, 1975). Nucleotide sequence analysis of MDV-1(+) RNA that had been modified by these reagents permitted the identification of cytidines that are located in exposed single-stranded regions. From these data, we propose a secondary structure for this RNA.

Sodium bisulfite modification creates many base-substitution mutations in MDV-1(+) RNA. We tested the effect of these mutations on template activity. Using a technique that prevents modification from occurring in a small region of the RNA, we located one or more mutations at the 3′ end of the template that are lethal to an early step in replication. We also discuss a technique for localized mutagenesis that will enable us to determine the effect of other base substitutions on particular replicative functions.

Experimental Procedures

Materials. Pancreatic ribonuclease A was purchased from Worthington. Ribonuclease T_1 was purchased from Calbiochem. Ribonucleoside 5′-[α-^{32}P]triphosphates were obtained from ICN. Methoxyamine hydrochloride, *N,N,N′,N′*-tetramethylenediamine, bromophenol blue, and xylene cyanol are products of Eastman Organic Chemicals. Sodium bisulfite was purchased from Mallinckrodt. Acrylamide and *N,N′*-methylenebis(acrylamide) were purchased from Bio-Rad.

Synthesis of 3′-Deoxyribonucleoside 5′-Triphosphates. 3′-Deoxyuridine was synthesized from 2,2′-anhydro-1-(β-D-arabinofuranosyl)uracil (Sigma) by the procedure of Brown et al. (1958), converted to a 5′-monophosphate by the method of Yoshikawa et al. (1967), and then converted to the 5′-triphosphate (3′-dUTP) by the method of Hoard & Ott (1965). 3′-Deoxyadenosine 5′-triphosphate (3′-dATP) was prepared by converting cordycepin (Sigma) to a 5′-monophosphate and then to a triphosphate as described above.

Qβ replicase was isolated from Qβ bacteriophage infected *Escherichia coli* Q13 by the procedure of Eoyang & August (1971), with the hydroxylapatite step omitted. Concentrations of replicase were calculated by assuming that A_{280} of a 1 mg/mL solution of Qβ replicase in a 1-cm path length spectrophotometer cell is 0.65 (Kamen, 1972) and that the molecular weight of Qβ replicase is 205 000. [The molecular weight was calculated by assuming subunit molecular weights of 35 000 for T_s and 45 000 for T_u (Kamen, 1969), 65 000 for S1 (Laughrea & Moore, 1977), and 60 000 for the viral subunit.]

Synthesis of MDV-1(+) RNA. MDV-1 RNA was synthesized in 1-mL reactions in which the final concentrations of the components were 12 mM $MgCl_2$, 84 mM Tris-HCl (pH 7.4), 400 μM (each) ribonucleoside triphosphates (one of which was isotopically labeled), 50–500 pg [(7–70) × 10^{-16} mol] of MDV-1 RNA, and 50–250 μg (0.25–1.25 nmol) of Qβ replicase. Reactions were incubated at 37 °C for 2 h. The 100–250 μg of MDV-1(+) RNA and MDV-1(−) RNA that was synthesized was isolated (Kramer et al., 1974), and MDV-1(+) RNA was separated from MDV-1(−) RNA as previously described (Mills et al., 1978).

Bisulfite Modification. [^{32}P]MDV-1(+) RNA was incubated for various lengths of time (1–38 h) at 25 °C in 50–500 μL of 3 M sodium bisulfite (pH 5.6). The RNA was subsequently freed of unreacted bisulfite and brought to pH 9.8 by chromatography on a 0.4 × 10 cm Sephadex G-50 (Pharmacia) column equilibrated in 20 mM Tris-base (pH 9.8), 3 mM EDTA, and 200 mM NaCl. The excluded RNA was

pooled and incubated in the same buffer at 25 °C for 6 h to allow conversion of the 5,6-dihydrouridine-6-sulfonate residues to uridine. The conditions for removal of the sulfonate moiety from the pyrimidine ring are a modification of those used by Goddard & Schulman (1972). The RNA was then precipitated in the presence of 400 mM NaCl and 2 volumes of ethanol. The RNA was purified by electrophoresis on a 7% polyacrylamide slab gel cast and run in 50 mM Tris–borate (pH 8.3) and 3 mM EDTA (Maniatis et al., 1975). Intact RNA was located by autoradiography with Kodak X-Omat R Film and was recovered from the gel by elution in 400 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 3 mM EDTA, followed by ethanol precipitation.

Methoxyamine Modification. [^{32}P]MDV-1(+) RNA was dissolved in 20 μL of 3 M methoxyamine (pH 5.5) and 5 mM $MgCl_2$, sealed in a capillary tube, and incubated at 37 °C for 20 h (Chang, 1973). The reaction was then layered over a 0.4 × 10 cm Sephadex G-50 column equilibrated with 20 mM Tris-HCl (pH 7.5), 400 mM NaCl, and 3 mM EDTA. The excluded RNA was recovered by ethanol precipitation.

Nucleotide Sequence Analysis. [^{32}P]MDV-1 RNA was sequenced by two-dimensional, high-voltage electrophoresis of its RNase T_1 and RNase A digestion products (Sanger et al., 1965). The first dimension buffer was 400 mM ammonium formate (pH 3.5) and 3 mM EDTA, and the cellulose acetate strips (Schleicher and Schuell) were soaked in the same buffer containing 7 M urea (C. Woese, personal communication).

Assay for Template Activity. This assay measures the ability of a template to direct the synthesis of a product strand through the first uridine residue. A 10–100-ng (0.14–1.4-pmol) amount of [^{32}P]MDV-1(+) RNA was heated to 100 °C for 1 min in 150 μL of 1 mM EDTA–NaOH (pH 7.0). The solution was then chilled to 0 °C and brought to 250 μL containing 84 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, and 31 μg (0.15 nmol) of Qβ replicase. This reaction mixture was incubated at 37 °C for 3 min. The temperature was then adjusted to 4 °C, and 1 min later GTP and ATP were added to a final concentration of 120 μM. One minute later, CTP and 3′-dUTP were added to a final concentration of 40 and 800 μM, respectively, and incubation was continued at 4 °C for 40 min. Since the chain-terminating nucleotide, 3′-dUTP, was present, rather than UTP, the synthesis of MDV-1(−) RNA product ceased with the incorporation of the first uridine residue. The reaction was then chilled to 0 °C and brought to 400 mM NaCl and 20 mM EDTA. This mixture was deproteinized by phenol extraction, a procedure which causes nascent product chains to form stable duplex structures with their RNA templates (Feix et al., 1967). After purification by Sephadex G-50 chromatography and ethanol precipitation, the RNA was dissolved in 20 μL of 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 50 mM NaCl, containing 2 mg of sucrose and 4 μg of xylene cyanol. The RNA was then analyzed by electrophoresis on a 7% polyacrylamide slab gel cast and then run at 250 V in 50 mM Tris–borate (pH 8.3) and 3 mM EDTA. The electrophoresis buffer was recirculated to avoid pH changes, and the gel was maintained at 15 °C. The template–product hybrid (with a mobility of 0.83 relative to xylene cyanol) migrated more slowly than uncomplexed MDV-1(+) RNA (with a relative mobility of 0.88). The two RNA bands were located by autoradiography and excised from the gel. The amount of ^{32}P in each band was determined by Cerenkov counting. The fraction of the total RNA in the gel that was in the form of a hybrid was used as a measure of the fraction of active template molecules present in the RNA population.

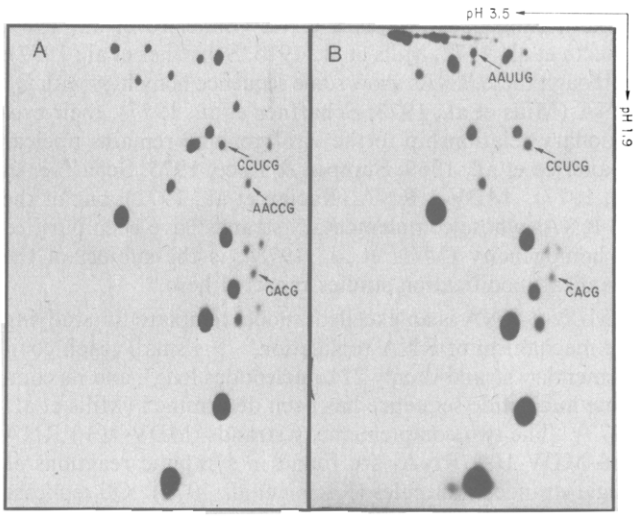

FIGURE 1: RNase T_1 fingerprint patterns of [α-^{32}P]GTP-labeled MDV-1(+) RNA. (A) Unmodified MDV-1(+) RNA. (B) MDV-1(+) RNA that was treated with sodium bisulfite for 38 h.

Preparation of "Masked" MDV-1(+) RNAs. A 250-μL reaction containing 84 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 3 μg (42 pmol) of [^{32}P]MDV-1(+) RNA, 23 μg (0.1 nmol) of Qβ replicase, 200 μM GTP, and 10 μM ATP was incubated at 37 °C for 3 min. The reaction was then chilled to 4 °C and brought to 200 μM CTP, 200 μM UTP, and 1 mM 3′-dATP (a 100:1 ratio of chain terminator to normal ATP). Incubation was continued for 40 min at 4 °C, and synthesis was stopped by chilling the reaction to 0 °C and bringing it to 400 mM NaCl and 20 mM EDTA. This mixture was deproteinized by phenol extraction. The resulting template–product hybrids were purified by Sephadex G-50 chromatography and ethanol precipitation and then analyzed by electrophoresis under the same conditions described for the template activity assay. The complexes were recovered from the gel by elution in 400 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 3 mM EDTA, followed by ethanol precipitation.

Results

Bisulfite Modification. The conversion of cytidine to uridine does not alter RNase T_1 or RNase A cleavage sites. Thus, the modification of a cytidine is seen in a two-dimensional fingerprint pattern of the RNA as the absence of the original oligonucleotide (C form) that contained the cytidine and the presence of a new oligonucleotide (U form) that contains a uridine in place of that cytidine.

Figure 1 compares the fingerprint pattern obtained from an RNase T_1 digest of unmodified MDV-1(+) RNA with the pattern obtained from MDV-1(+) RNA that had been modified by exposure to bisulfite for 38 h. Some cytidine-containing oligonucleotides, such as CACG and CCUCG, were unaffected by bisulfite modification and were present in both patterns. Other oligonucleotides that contained cytidines were converted to oligonucleotides that contained uridines. For instance, the C form, AACCG, was replaced by the U form, AAUUG, in the fingerprint pattern of the modified RNA. Modification of MDV-1(+) RNA was essentially complete after 38 h of bisulfite treatment since longer modification times did not result in significant changes in the fingerprint patterns.

The time course of modification of several different oligonucleotides was observed by sequencing a series of MDV-1(+) RNA samples that were exposed to bisulfite for various times (1–12 h). Representative time courses are shown in Figure 2. The results demonstrate that the conversion of a cytidine to a uridine is a pseudo-first-order process, with a different

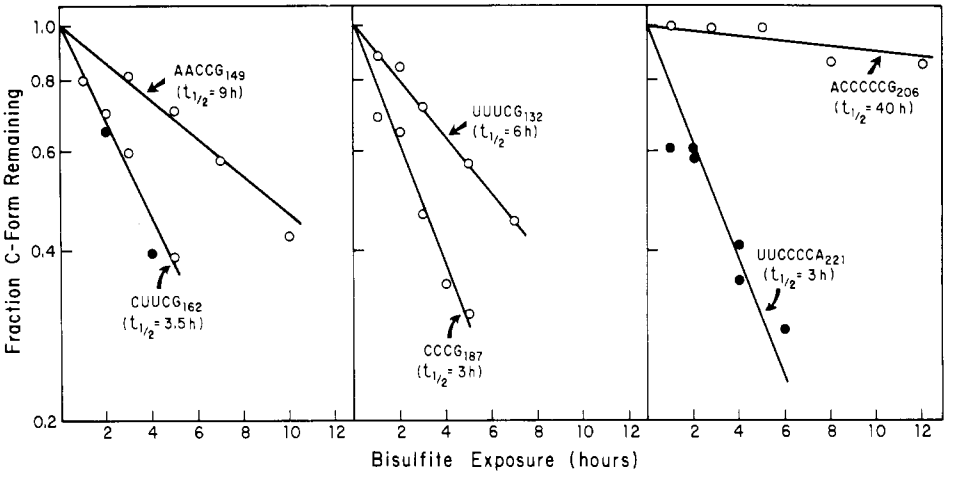

FIGURE 2: Kinetics of cytidine to uridine conversions that occur in MDV-1(+) RNA during bisulfite treatment. [^{32}P]MDV-1(+) RNA was incubated with bisulfite and sampled at various times. The RNA in each sample was digested with RNase T_1 and then analyzed by two-dimensional electrophoresis. At each time point, the fraction of strands that contained a particular C-form oligonucleotide was calculated from the ratio of the molar yield of that oligonucleotide in a fingerprint pattern of modified RNA to the molar yield of the same oligonucleotide in a fingerprint pattern of unmodified RNA. Open circles (○) represent data obtained from [α-^{32}P]GTP-labeled MDV-1(+) RNA; closed circles (●) represent data obtained from [α-^{32}P]UTP-labeled MDV-1(+) RNA. The numerical subscript at the 3′ nucleotide of each oligonucleotide identifies the position of that nucleotide in the sequence of MDV-1(+) RNA (see Figure 3). These semilogarithmic plots indicate that bisulfite modification is a pseudo-first-order process, with a rate constant that is different for different oligonucleotides.

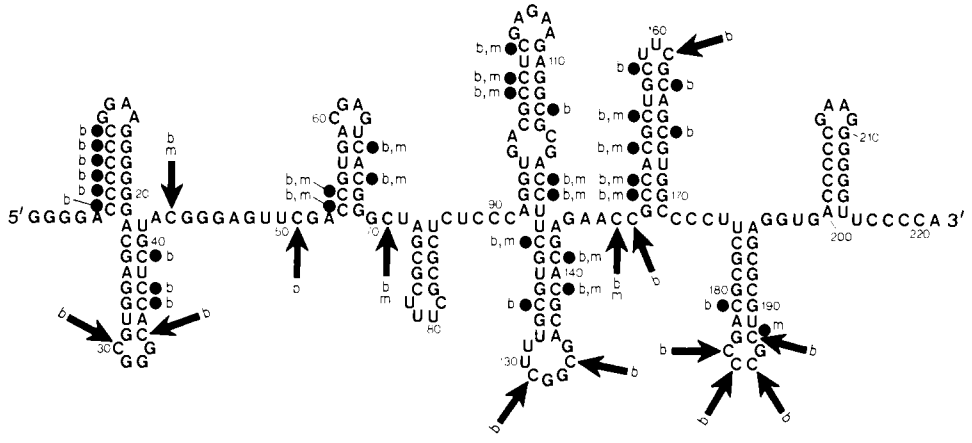

FIGURE 3: Nucleotide sequence of MDV-1(+) RNA, showing the modification results. The sequence is arranged to show the hairpin structures that are likely to be present. Lower case letters indicate the modification agent used: bisulfite (b); methoxyamine (m). Arrows (→) identify cytidines that are susceptible to modification, and closed circles (●) identify those that are not susceptible. Only those cytidines whose individual susceptibility to modification could be determined by the criteria described under Results are labeled. The kinetic results indicate that it is likely that one or more cytidines in the 3′-terminal oligonucleotide, $UUCCCCA_{221}$, were modified by bisulfite, although they are not shown as modified in this figure because we could not determine which of the four cytidines was susceptible. The secondary structure shown is the one most compatible with the modification data and with theoretical calculations of the stability of each hairpin (Tinoco et al., 1973). The sequence shown contains corrections to the sequence published previously (Mills et al., 1973): two additional cytidines occur at nucleotide positions 90 and 173, a terminal adenosine occurs at position 221, and an adenosine is present at position 105.

rate constant for different oligonucleotides. If more than one cytidine to uridine conversion occurs within a given oligonucleotide, the observed rate constant is the sum of the rate constants associated with the disappearance of each cytidine. Among the few instances where the rate of a single cytidine to uridine conversion could be followed, the shortest half-life was 3.5 h. In general, the half-lives of oligonucleotides that were modified ranged from 3 to 10 h.

Nucleotide sequence analysis of MDV-1(+) RNA that had been treated with bisulfite for 38 h allowed the susceptibility of 43 of the 76 cytidines to be determined. These data are shown in Table I. If the data indicated that a particular cytidine residue was converted to uridine in more than 65% of the RNA strands examined, it was scored as modified; if less than 35% was converted, it was scored as unmodified. By these criteria, 13 cytidines were modified and 30 cytidines were not modified. In addition, the susceptibilities of the cytidines at positions 155 and 188 were established in an experiment that will be described later. Figure 3 summarizes these results.

The reactivities of the other 31 cytidines could not be determined for various reasons: the cytidines in ACG_{24} and ACG_{61} and the two cytidines in $CUUUCG_{82}$ fell just short of being scored as modified, and the five cytidines in $ACCCCCG_{206}$ fell just short of being scored as unmodified. The fate of each of the 14 cytidines in $CUCUCCCAG_{93}$, $CCCCUUCG_{178}$, and $UUCCCCA_{221}$ could not be determined because the U forms of these oligonucleotides were obscured by the presence of other oligonucleotides in the fingerprint pattern. The reactivities of the remaining eight cytidines could not be determined because they are located in small oligonucleotides that frequently recur in the sequence of MDV-1 RNA.

Methoxyamine Modification. To complement the bisulfite modification experiments, we modified MDV-1(+) RNA with methoxyamine. Oligonucleotides that contain cytidine–methoxyamine adducts migrate in a fingerprint pattern as though

Table I: Molar Yield of Oligonucleotides from RNase T_1 and RNase A Digests of Bisulfite-Treated MDV-1(+) RNA[a]

expected yield of C form in unmodified RNA	C form	yield of C form labeled with CTP	GTP	ATP	UTP	U form	yield of U form labeled with CTP	GTP	ATP	UTP	susceptible and resistant cytidines
0.75	$ACCCCCCG_{12}$	≥0.5	≥0.5	–	–	–	–	–	–	–	6, 7, 8, 9, 10, 11
1.0	ACG_{24}	≤0.5	–	≤0.5	–	AUG_{24}	≥0.6	–	≥0.5	–	
1.2	CG_{31}	–	≤0.1	–	–	UG_{31}	–	1.0	–	–	30
0.85	$GGGC_{34}$	≤0.1	0.0	–	–	$GGGU_{34}$	≥0.6	≥0.75	–	–	34
1.1	AC_{36}	1.0	–	–	–	AU_{36}	0.0	–	–	–	36
0.9	$CACCUCG_{40}$	0.0	0.1	0.1	0.15	$UACCUCG_{40}$	0.6	0.7	0.7	0.85	34, 36, 37, 39
1.1	$UACG_{44}$	0.0	≤0.1	≤0.2	–	$UAUG_{44}$	0.6	0.7	1.0	–	43
1.1	$UUCG_{52}$	0.0	0.0	0.1	0.1	$UUUG_{52}$	–	–	1.1	0.95	51
0.95	$ACCG_{56}$	0.65	0.7	–	0.65	$AUCG_{56}$	0.1	0.1	–	0.1	54, 55
1.0	ACG_{61}	≤0.5	–	≤0.5	–	AUG_{61}	≥0.6	–	≥0.5	–	
0.95	$UCACG_{68}$	0.65	0.6	0.8	–	$UUACG_{68}$	0.2	0.2	0.2	–	65, 67
0.85	$GGGC_{71}$	≤0.1	0.0	–	–	$GGGU_{71}$	≥0.6	≥0.75	–	–	71
0.95	$CUAG_{74}$	0.0	≤0.2	≤0.2	0.1	$UUAG_{74}$	0.9	0.9	1.0	0.9	71
1.0	$CUUUCG_{82}$	0.0	0.0	–	0.0	$UUUUUG_{82}$	>0.5	>0.5	–	>0.5	
1.0	GC_{100}	1.0	–	–	–	GU_{100}	–	–	–	–	100
0.95	$CCUCG_{104}$	0.8	1.0	1.1	1.0	–	–	–	–	–	100, 101, 103
0.95	$GAGAAGAGGC_{113}$	1.0	1.0	–	–	$GAGAAGAGGU_{113}$	–	0.0	–	–	113
1.1	$ACCUUCG_{123}$	≥0.6	1.1	–	1.0	–	–	–	–	–	118, 119, 122
1.1	CG_{127}	–	–	–	1.1	UG_{127}	–	–	–	0.0	126
0.95	$UUUCG_{132}$	0.0	0.0	–	0.0	$UUUUG_{132}$	0.8	1.1	–	0.8	131
1.3	GGC_{134}	0.2	0.0	–	–	GGU_{134}	1.0	0.95	–	–	134
1.0	$CACG_{142}$	0.8	1.1	0.95	–	–	–	–	–	–	139, 141
1.0	$GAGAAC_{147}$	0.15	0.2	–	–	$GAGAAU_{147}$	0.9	0.8	–	–	147
0.8	$AACCG_{149}$	0.0	0.1	0.1	–	$AAUUG_{149}$	0.8	0.9	0.8	–	147, 148
1.0	GC_{150}	1.1	–	–	–	GU_{150}	–	–	–	–	150
0.95	$CCACG_{154}$	0.8	0.9	1.0	–	–	–	–	–	–	150, 151, 153
1.0	$CUUCG_{162}$	0.0	0.0	–	0.0	$CUUUG_{162}$	0.8	1.0	–	0.9	158, 161
1.1	CAG_{165}	–	–	–	–	UAG_{165}	≤0.3	≤0.3	≤0.3	–	163
1.1	CG_{167}	–	–	–	1.1	UG_{167}	–	–	–	0.0	166
1.1	CAG_{183}	–	–	–	–	UAG_{183}	≤0.3	≤0.3	≤0.3	–	181
1.0	AGC_{184}	0.0	–	–	–	AGU_{184}	1.2	1.2	–	–	184
1.1	$CCCG_{187}$	0.05	0.0	–	–	$UUUG_{187}$	0.85	–	–	–	184, 185, 186
0.75	$ACCCCCG_{206}$	≥0.5	≥0.5	≥0.2	–	–	–	–	–	–	
0.5	$UUCCCCA_{221}$	–	–	–	0.15	–	–	–	–	–	

[a] Four MDV-1(+) RNA preparations, each labeled by a different α-^{32}P-labeled ribonucleoside triphosphate, were modified by bisulfite for 38 h and examined by nucleotide sequence analysis. Since oligonucleotides that contain only adenosines and guanosines were unaffected by bisulfite, the radioactivity of these oligonucleotides was used as a standard against which the molar yield of the other oligonucleotides was determined. The molar yield of each C-form oligonucleotide is listed on the same line as its related U-form oligonucleotide. A dashed line indicates that the oligonucleotide was not labeled or that it was not uniquely discernible in the fingerprint pattern. To calculate the percentage of strands modified at a particular cytidine, we compared the molar yield of each C and U form with the molar yield obtained for that C form when unmodified RNA was analyzed (shown in the left-hand column). The sum of the molar yields of related C and U forms should equal the expected molar yield. In a few instances where more than one cytidine was present in an oligonucleotide, the sum of the C form and the U form was lower than expected, because more than one U form occurred, and these other U forms could not be detected unambiguously. However, knowledge of the sequence of the predominant U forms, in conjunction with the molar yields listed in the table, enabled us to draw conclusions about the susceptibility to modification of 43 cytidines in the sequence. These cytidines are identified in the right-hand column by their position in the sequence. The underlined numbers identify the modified cytidines.

the modified cytidines were uridines (Cashmore et al., 1971; Chang, 1973), resulting in a displacement of these oligonucleotides from their normal positions. We used the molar yields of the unmodified oligonucleotides in a fingerprint pattern of the modified RNA to measure the degree of modification. Table II summarizes these results. Percent modification represents the cumulative effect of modification on all the cytidines within an oligonucleotide.

The cytidines that were clearly resistant or clearly susceptible to modification are indicated in Figure 3. A comparison of the susceptibility of the cytidines to bisulfite and to methoxyamine indicates that, in general, both reagents attack the same sites.

Template Activity of Bisulfite-Modified RNA. In order to test the template activity of bisulfite-modified MDV-1(+) RNA, we developed a procedure that permits the electrophoretic separation of active and inactive templates. In this assay, [^{32}P]MDV-1(+) RNA is incubated with an excess of Qβ replicase to allow enzyme–template complexes to form. GTP and ATP are then added, permitting product strand synthesis to proceed until the first cytidine is required. Finally, CTP and the chain terminator, 3′-dUTP, are added, and incubation is continued until all product chain synthesis has ceased due to the incorporation of the terminator. The products of this reaction are 13 nucleotides long. Terminated product chains do not dissociate from the replication complex during incubation (Kramer & Mills, 1978). The reaction mixture is then deproteinized with phenol, resulting in the formation of a stable base-paired complex between each product strand and its template. These complexes are then

Table II: Modification of Oligonucleotides in Methoxyamine-Treated MDV-1(+) RNA[a]

oligonucleotide	% modifcn	oligonucleotide	% modifcn
$UACG_{44}$	73	$CACG_{142}$	0
$ACCG_{56}$	0	$GAGAAC_{147}$	90
$UCACG_{68}$	16	$CCACG_{154}$	0
$CUAG_{74}$	68	CUG_{157} and CUG_{190}	18
$CUUUCG_{82}$	80	$CUUCG_{162}$	50
$CCUCG_{104}$	0	$CCCG_{187}$	82
$ACCUUCG_{123}$	16		

[a] [α-^{32}P]GTP-labeled MDV-1(+) RNA was treated with methoxyamine, digested with either RNase T_1 or RNase A, and then analyzed by two-dimensional electrophoresis. Percent modification was calculated as 100 times the difference between the molar yield of an oligonucleotide in an unmodified MDV-1(+) RNA control digest and the molar yield of the same unmodified oligonucleotide in a digest of modified RNA, divided by the molar yield of the oligonucleotide in unmodified RNA.

separated from unused templates by acrylamide slab gel electrophoresis. Control experiments indicated that the product chains remain hybridized to their templates during electrophoresis. Other control experiments showed that heat-denatured product–template hybrids do not reassociate under the conditions of synthesis, isolation, and electrophoresis. We were therefore able to define template activity as the percent of the total MDV-1(+) RNA that is isolated as a hybrid. When unmodified MDV-1(+) RNA was assayed for template activity, 50–70% of the RNA strands were isolated in this form. In contrast, MDV-1(+) RNA that had been modified by bisulfite treatment for 38 h was completely inactive, indicating that it was unable to direct (−) strand synthesis.

In order to determine whether this loss of template activity occurs with the same time course as the modification of particular cytidines, we examined the loss of template activity during bisulfite treatment. Samples of MDV-1(+) RNA were exposed to bisulfite for various lengths of time (1–5 h) and then assayed for template activity. A plot of the log of template activity vs. time showed that the loss of template function during bisulfite treatment is a first-order process with a half-life of 2.3 h. This rapid inactivation occurred at a rate that is similar to the rate at which several oligonucleotides were modified by bisulfite. One of these rapidly modified oligonucleotides is the 3′-terminal oligonucleotide $UUCCCCA_{221}$ (see Figure 2).

Preparation of Selectively Modified RNA. To see if the modification of one or more of the cytidines in the 3′-terminal oligonucleotide was responsible for the loss of template activity, we prepared an MDV-1(+) RNA that was completely modified except for the cytidines in the 3′-terminal oligonucleotide. This was accomplished by synthesizing a short MDV-1(−) strand product on an MDV-1(+) strand template and isolating the template–product hybrid. Since cytidines in a double-stranded conformation are not modified by bisulfite, the presence of this (−) strand mask prevented modification at the 3′ end. After modification, the mask was removed and the selectively modified RNA was assayed for template activity. This strategy is outlined below and illustrated in Figure 4.

Masked MDV-1(+) RNAs were prepared in a two-stage reaction (Billeter et al., 1969). In the first stage, only GTP and ATP were present, permitting synthesis of MDV-1(−) RNA to proceed until the first cytidine was required. In the second stage, CTP, UTP, and the chain terminator, 3′-dATP, were present. Since we used a ratio of 3′-dATP to ATP that results in an equal probability of incorporation at a given site (Kramer & Mills, 1978), half of the product chains were terminated where the first adenosine was required, half of the

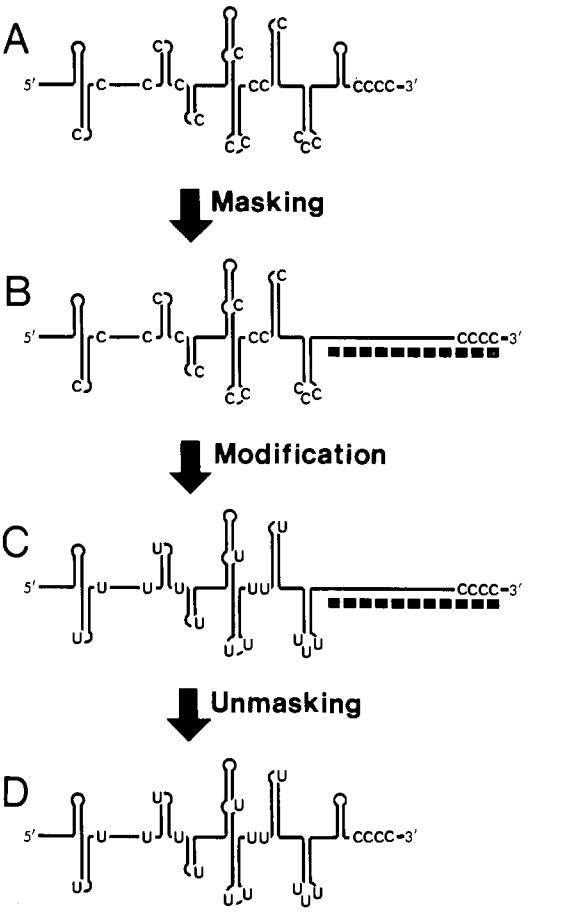

FIGURE 4: Use of a mask to selectively modify RNA. MDV-1(+) RNA (A) is used as a template for Qβ replicase in a reaction that contains one of the 3′-deoxyribonucleoside triphosphate chain terminators. During isolation, the terminated product strand (shown as a dashed line) forms a hybrid with the 3′ end of its MDV-1(+) RNA template, and as a result the cytidines at the 3′ end of the template are placed in a double-stranded conformation (B). When this hybrid is treated with bisulfite, the cytidines covered by the mask are protected from modification (C). The mask is then removed by heat denaturation, resulting in a selectively modified MDV-1(+) RNA (D) that can be tested for template activity.

unterminated chains were terminated at the next adenosine, and so on. The template–product hybrids that were formed during isolation of the RNA were separated from one another by acrylamide slab gel electrophoresis. Ninety-five percent of these template–product hybrids were found in three bands in the gel. The length of the product strands in each band was determined by nucleotide sequence analysis. The three major products were 23, 32, and 45 to 46 nucleotides in length. The 23-nucleotide mask was hybridized to residues 198–220 of MDV-1(+) RNA, the 32-nucleotide mask covered residues 189–220, and the 45 to 46-nucleotide mask covered residues 175– and 176–220. These hybrids are stable during electrophoresis and isolation (see Figure 5).

Figure 6 shows the RNase T_1 fingerprint pattern of [^{32}P]-MDV-1(+) RNA that was modified by bisulfite while hybridized to the 45- to 46-nucleotide mask. The results indicate that all of the cytidines that were shown to be modified in an unmasked control RNA were also modified in the masked RNA, except for the cytidines covered by the mask. Thus, the presence of the mask does not perturb the unmasked portion of the molecule in a manner that alters the sensitivity of the unmasked cytidines. Table III shows further data that demonstrate that the presence of a mask leads to the selective modification of the RNA. In particular, the 23-nucleotide

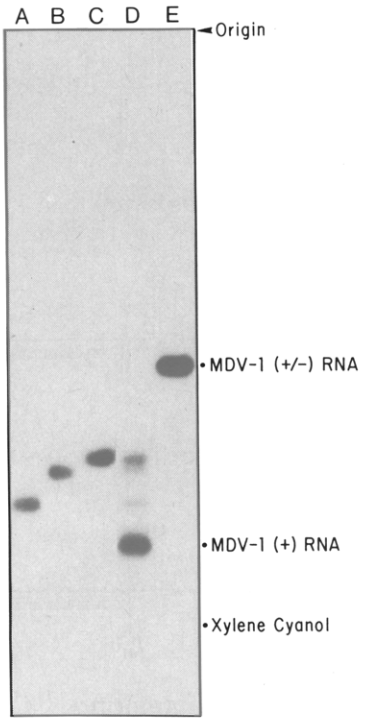

FIGURE 5: Electrophoretic mobilities of masked RNAs. Masked [^{32}P]MDV-1(+) RNAs were separated from one another by acrylamide slab gel electrophoresis and then isolated from the gel for use in bisulfite modification experiments. In order to demonstrate that these hybrids are stable under the conditions of isolation, we took samples of each masked RNA and again analyzed them by electrophoresis. An autoradiograph of the resulting gel is shown. Track A: MDV-1(+) RNA hybridized to the 23-nucleotide mask. Track B: MDV-1(+) RNA hybridized to the 32-nucleotide mask. Track C: MDV-1(+) RNA hybridized with the 45- to 46-nucleotide mask. Track D: mixture of unhybridized MDV-1(+) RNA and the RNA hybrids present in tracks A–C. Track E: MDV-1(+) RNA/MDV-1(–) RNA hybrid. The absence of unhybridized MDV-1(+) RNA in tracks A–C indicates that the masked RNAs were stable during electrophoresis and isolation.

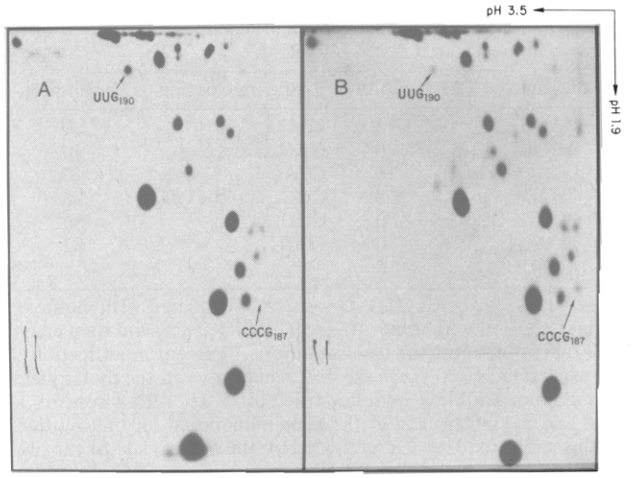

FIGURE 6: Effect of the presence of a mask on the modification of MDV-1(+) RNA. A solution of [α-^{32}P]GTP-labeled MDV-1(+) RNA hybridized with the 45- to 46-nucleotide mask was split into two aliquots. The RNA in one aliquot was heat denatured to dissociate the mask from the MDV-1(+) RNA, by dissolving the RNA in 1 mM EDTA–NaOH (pH 7) and incubating it at 100 °C for 30 s, followed by chilling to 0 °C. Both this RNA and the masked RNA in the other aliquot were treated with bisulfite for 38 h. [Preliminary experiments showed that dissociated masks do not reanneal to the MDV-1(+) RNA during bisulfite treatment.] After modification, the mask was dissociated from the RNA in the second aliquot by heat denaturation. The RNA in each sample was then digested with RNase T_1 and analyzed by two-dimensional electrophoresis. (A) Fingerprint pattern of MDV-1(+) RNA that was not protected by a mask during bisulfite treatment. (B) Fingerprint pattern of MDV-1(+) RNA that was protected by a mask during bisulfite treatment. An analysis of the differences between the two fingerprint patterns confirmed that the cytidines covered by the masks were not modified. For example, $CCCG_{187}$ was not present in pattern A because one or more of its cytidine residues was modified. However, it was present in pattern B because it is located in the region of MDV-1(+) RNA that was protected by the mask.

mask protected only the cytidines in the 3′-terminal oligonucleotide, $UUCCCCA_{221}$.

Template Activity of Selectively Modified RNA. To study the effect that modification of the 3′-terminal cytidines has on template activity, we exposed MDV-1(+) RNA protected by the 23-nucleotide mask to bisulfite and sampled the RNA during the course of treatment. The mask was then removed from the RNA in each sample by heat denaturation, and the RNA was assayed for template activity. Figure 7 shows the results of this experiment. Bisulfite treatment had no effect on the template activity of the selectively modified RNA. Yet, unmasked RNA samples that were taken from a control reaction lost activity during the course of bisulfite treatment. These results demonstrate that the modification of one or more cytidines in the 3′-terminal oligonucleotide is responsible for the loss of template activity.

Discussion

Conformational Analysis. MDV-1(+) RNA is a highly structured molecule as evidenced by its limited susceptibility to modification by sodium bisulfite and methoxyamine. Of the 45 cytidines whose susceptibility to bisulfite could be individually determined, only 14 were modified. Figure 3 shows a secondary structure model of MDV-1(+) RNA that positions resistant cytidines in double-helical structures and susceptible cytidines in single-stranded regions. The hairpins shown are very stable, with calculated free energies of formation (Tinoco et al., 1973) ranging from –8 to –21 kcal/mol. Even though we could not interpret resistance to modification in terms of stacking or tertiary interactions, we feel confident that many of the secondary structures shown are elements of the RNA's actual conformation.

Since the modification data are based on the molar yield of oligonucleotides rather than of individual cytidines, the susceptibility of 31 cytidines could not be individually determined. However, the limited data obtained for these cytidines are generally consistent with the secondary structure shown in Figure 3. For instance, at least one of the two cytidines in $CUUUCG_{82}$ was definitely modified by bisulfite. We think it likely that the cytidine at position 81, which is placed in a single-stranded region in Figure 3, is modified. In general, the susceptibility of individual cytidines could not be determined in instances where more than one cytidine occurs in the same oligonucleotide. However, the susceptibility of the cytidines in $ACCCCCG_{206}$ and $UUCCCCA_{221}$ can be inferred, because the half-life of $UUCCCCA_{221}$ during bisulfite treatment was only 3 h, while the half-life of $ACCCCCG_{206}$ was 40 h (see Figure 2). It is therefore likely that, as shown in Figure 3, the 3′-terminal oligonucleotide, $UUCCCCA_{221}$, is at least partially single stranded, and $ACCCCCG_{206}$ is not.

The kinetic results illustrated in Figure 2 show that susceptibility actually varies from cytidine to cytidine. For example, the cytidine at position 131 had a half-life of 6 h, while the cytidine at position 161 had a half-life of only 3.5 h. Since both of these cytidines probably occur in single-stranded regions, these observations imply that the kinetics of modification are influenced by subtle aspects of conformation, such as stacking interactions, tertiary structures, and perhaps the

Table III: Effect of the Presence of a Mask on the Molar Yield of Some Oligonucleotides from Bisulfite-Treated RNAs[a]

	oligonucleotide	unmodified	modified	masked, modified
A:	$CUUCG_{162}$	1.2	0.1	0.1
	$CCCG_{187}$	1.0	0.0	0.9
	CUG_{157} and CUG_{190} [b]	2.0	1.0	1.7
B:	$CUUCG_{162}$	1.1	0.1	0.2
	$UUCCCCA_{221}$	0.5	0.1	0.5

[a] Two masked MDV-1(+) RNAs were studied. The data in section A of the table were obtained from [α-^{32}P]GTP-labeled RNA hybridized to the 45- to 46-nucleotide mask, and the data in section B were obtained from [α-^{32}P]UTP-labeled RNA hybridized to the 23-nucleotide mask. Both masked RNAs were treated as follows. The solution containing the RNA was split into three aliquots. The RNA in the first aliquot was not treated with bisulfite and served as an unmodified control. The RNA in the second aliquot was heat denatured to dissociate the mask from the MDV-1(+) RNA. Both this RNA and the masked RNA in the third aliquot were treated with bisulfite for 38 h. Thus, the RNA in the second aliquot served as a modified control for comparison with the masked, modified RNA. The RNA in all three aliquots was then heat denatured, digested with RNase T_1, and analyzed by two-dimensional electrophoresis. The molar yields of some of the oligonucleotides present in the resulting fingerprint patterns are listed in this table. $CUUCG_{162}$, labeled in all of the fingerprint patterns, is a control oligonucleotide, since it was not protected by either mask and was always modified. On the other hand, $CCCG_{187}$ was protected from modification when it was covered with the 45- to 46-nucleotide mask. When $UUCCCCA_{221}$ was covered, it was also not modified. [b] The results of this experiment also show that the cytidine at position 188 can be modified while the cytidine at position 155 cannot. Although 1 mol of CUG was modified in unmasked RNA, virtually no modification of CUG was observed when one of the two CUG oligonucleotides was covered with a mask, indicating that CUG_{190} contained the modified cytidine. This result is illustrated in Figure 6 by the absence of UUG_{190} in the fingerprint pattern of masked, modified MDV-1(+) RNA.

identity of neighboring nucleotides. These subtle conformational differences may well be reflected in the modification of the cytidines that we have positioned in loop-closure base pairs in Figure 3. Although the cytidines at positions 11, 103, and 158 were not modified by bisulfite, those at 34, 188, and possibly 77 were. Interestingly, the cytidine at position 188 was not modified by methoxyamine. While we have placed cytidines at positions 34, 77, and 188 in loop-closure base pairs to maximize the secondary structure stability of the hairpin helixes, the detailed conformational state is not completely clear from our results.

MDV-1(+) RNA may occur in more than one conformation. The presence of molecules with alternate conformations would explain the partial susceptibility of the cytidine at position 23 and the less than complete modification of some oligonucleotides (see Tables I and II). It is also possible that some early modifications alter the conformation of the RNA, exposing some initially protected cytidines or protecting some otherwise susceptible cytidines. Indeed, the electrophoretic mobility of the RNA did change during the course of bisulfite treatment (data not shown), raising the possibility that a conformational change was occurring.

We are confident, however, that the modification results, indicated by the arrows and filled circles in Figure 3, reflect many of the structural features of unmodified MDV-1(+) RNA. This view is supported by experiments with $tRNA^{Phe}$ that show an excellent agreement between the chemical modification results and the conformation revealed by X-ray crystallography [Robertus et al., 1974; Rhodes, 1975; see Rich & RajBhandary (1976) for a review]. In summary, we conclude that it is likely that most of the hairpin structures shown in Figure 3 actually occur in MDV-1(+) RNA.

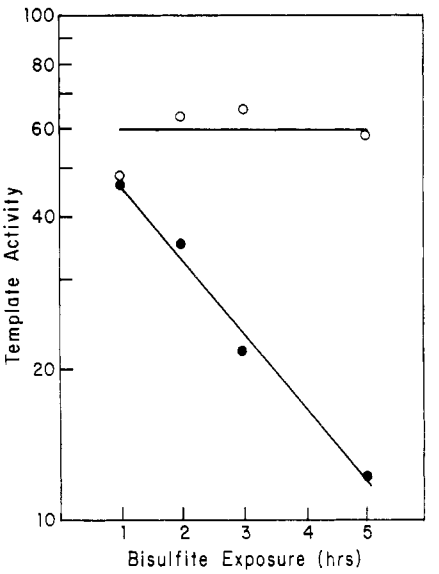

FIGURE 7: Effect of the modification of one or more cytidines in the 3′-terminal oligonucleotide of MDV-1(+) RNA on its template activity. A solution of MDV-1(+) RNA hybridized to the 23-nucleotide mask was split into two aliquots. The RNA in one aliquot was heat denatured to dissociate the mask from the MDV-1(+) RNA. Both this RNA and the masked RNA in the other aliquot were incubated with bisulfite. Samples of each reaction were taken at various times, and the RNA in each sample was isolated. RNA samples from the reaction containing masked RNA were then heat denatured. The RNA in each sample was then assayed for template activity. (The presence of the dissociated mask has no effect on the template activity.) The log of the template activity of the RNA obtained from each reaction is plotted against the length of bisulfite treatment. The unmasked MDV-1(+) RNA (●) lost template activity, while the template activity of masked MDV-1(+) RNA (○) remained equal to the template activity of unmodified MDV-1(+) RNA (50–70%).

Consequences of Nucleotide Substitutions on Replication. The results of the template activity assay indicated that one or more of the nucleotide substitutions caused by bisulfite modification had a lethal effect on an early stage of replication. Since the template activity of selectively modified RNA was unaffected by bisulfite treatment (see Figure 7), the modification of one or more of the cytidines in the 3′-terminal oligonucleotide was lethal. We think it likely that this modification is lethal because of changes in the nucleotide sequence rather than alterations of template conformation. Since we have shown previously that MDV-1(+) RNA that had been modified by bisulfite for 48 h can bind to Qβ replicase (Mills et al., 1977), the results reported here support the view that a single-stranded cytidine-containing sequence at the 3′ end of the template is required for product chain initiation. A similar inference was drawn by Küppers & Sumper (1975), who investigated the template activity of various synthetic RNAs, and by Rensing & August (1969), who observed that the removal of a single cytidine from the 3′ end of Qβ RNA substantially reduced its template activity.

The template activity assay showed that RNA modified in the presence of a mask was able to direct the synthesis of the first 13 nucleotides of the product strand. Preliminary experiments indicate, however, that this selectively modified template is not able to compete with unmodified MDV-1 RNA in an autocatalytic reaction. It is therefore likely that some of the substitutions beyond the 13th nucleotide adversely affect the replicative process. Nucleotide substitutions may weaken replicase binding, lower the initiation rate, diminish the elongation rate, cause premature termination, or inhibit the dissociation of the replication complex. We feel it worthwhile

to study these detrimental nucleotide substitutions further by generating site-specific mutations and by assaying their effect on particular replicative functions.

Site-Directed Mutagenesis. The masking technique we used to protect the 3′ end of MDV-1(+) RNA from modification can be extended to direct a variety of sequence and conformational alterations. One or more masks can be hybridized to the RNA template to direct or prevent modification in a desired region. Mutations can be directed to any region of the molecule, including regions that form secondary structures. Consequently, this technique is useful for introducing structural alterations as well as nucleotide substitutions. The large collection of mutant RNAs that can be generated will enable us to explore, in detail, the interaction between Qβ replicase and its template.

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