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Synthesis of an amplifiable reporter RNA for bioassays

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ABSTRACT

The replacement of reporter groups, such as fluorescent molecules or enzymes, by an amplifiable reporter should lead to bioassays of greatly increased sensitivity, since a very large number of copies of the reporter can be accumulated in a short time. Midivariant RNA is an appropriate reporter, since it is autocatalytically replicated by Qβ RNA polymerase in vitro. This RNA can be amplified exponentially, with a population doubling time of 36 seconds, resulting in the synthesis of 10^6 copies of each molecule in 12 minutes. We have used chemical methods to attach biotin to the 5' terminus of midivariant RNA via a disulfide linker. This biotinylated RNA combines with avidin to give a product that is readily purified by gel electrophoresis. The RNA-biotin-avidin adduct, and the RNA released from it by reductive cleavage of the linker arm, replicate normally. The RNA-biotin-avidin adduct should be a suitable reporter for a variety of replication-assisted bioassays involving biotinylated antibodies or biotinylated nucleic acid probes.

INTRODUCTION

It is often necessary to detect and estimate a small amount of a specific biopolymer against a background of a much larger quantity of unrelated material. Some important cases are the assay of proteins present at very low levels in cell lysates, the counting of sparse receptors on cell surfaces, and the detection of short defined oligonucleotide sequences in total genomic DNA. The specificity of suitable assays usually depends on the use of a biopolymer that combines with the target with high affinity and has a low affinity for all other components of the sample -- for example, an antibody probe for a protein or a complementary oligonucleotide probe for a DNA sequence. The sensitivity of typical assays is achieved by attaching a reporter group to the probe. Typical reporters are fluorescent organic molecules and ^{32}P-labeled phosphate groups.

The sensitivity of nonradioactive assays has been improved by attaching an enzyme to the probe, often via a biotin-avidin linker (1). Then it is possible to accumulate a large number of product molecules for each enzyme-probe adduct, and hence to obtain sensitivities much greater than is possible using small

reporter molecules directly. Peroxidase and phosphatase, enzymes that are readily assayed by sensitive colorimetric methods, are widely used as reporters in this context (2).

A natural extension of these enzymatic assay methods would be the use of a replicating entity as the reporter. Exponential growth of the population of reporters would lead to the accumulation of a very large number of copies in a few replication cycles -- for example, 10^6 copies of each reporter molecule in 20 replication cycles. RNAs that are templates for Qβ replicase (3) seem to be the optimal reporters. One or a very small number of RNA molecules can serve as templates for autocatalytic amplification (4, 5), and doubling times of some RNAs are well under one minute (6).

We have recently described a simple chemical method of attaching biotin to the 5' phosphate of an oligodeoxynucleotide (7). We describe here an extension of this chemistry that permits us to attach biotin, via a cleavable linker, to the 5' end of midivariant RNA (8), a 221 nucleotide template that is autocatalytically replicated by Qβ replicase in vitro (Fig. 1). This adduct combines with avidin to give a product that is still a normal substrate for the replicase. We believe that the adduct can be used to develop a versatile amplifiable reporter system for a variety of bioassays.

MATERIALS AND METHODS

Enzymes and Chemicals

The following were purchased: calf intestine alkaline phosphatase (Boehringer Mannheim), bacteriophage T4 polynucleotide kinase (Pharmacia P-L Biochemicals); ribonuclease T_1 and highly polymerized yeast RNA (Calbiochem); N-hydroxysuccinimidobiotin (Sigma); 2,2'-dithiobis(ethylamine)dihydrochloride (C.T.C. Organics); 1-ethyl-3,3-dimethylaminopropyl carbodiimide (Aldrich); biotinylated agarose (binds 8 mg avidin/ml) and avidin DN (Vector Labs); and [γ-^{32}P]ATP and [α-^{32}P]GTP (Amersham). Qβ replicase was isolated from bacteriophage Qβ-infected Escherichia coli Q13 by the procedure of Eoyang and August (10), with the hydroxylapatite step omitted.

Preparation of MDV-1 (+) RNA

A readily identifiable mutant of midivariant RNA (MDV-1 RNA) that possesses three nucleotide substitutions (6) was used in these studies (Fig. 1). 758 μg of MDV-1 RNA were synthesized by incubating 705 ng mutant MDV-1 (+) RNA template and 69 μg Qβ replicase for 210 minutes at 37°C in 1 ml containing 1 mM ATP, 1 mM CTP, 1 mM GTP, 1 mM UTP, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5. The incubation mixture was then deproteinized by extraction with phenol (11) and

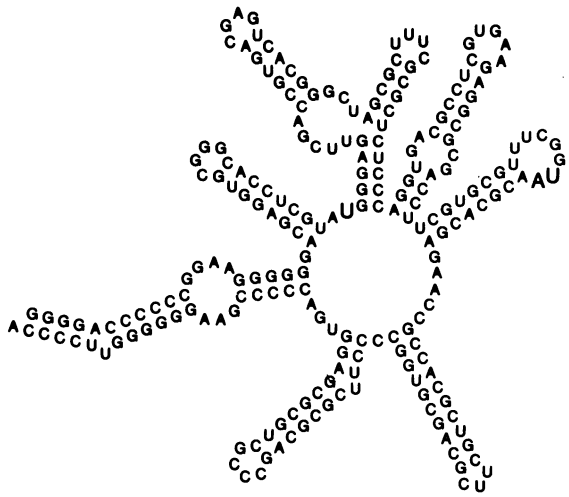


Figure 1: Nucleotide sequence of mutant MDV-1 (+) RNA. The RNA is folded into the secondary structure predicted by computer to be most stable (9). Large letters identify the three nucleotide substitutions that distinguish this sequence from wild-type MDV-1 (+) RNA.

the RNA was isolated by precipitation with 2 vol of ethanol at -20°C in the presence of 2 M ammonium acetate. MDV-1 (+) RNA was separated from MDV-1 (-) RNA by acrylamide gel electrophoresis in the presence of 1 mM $MgCl_2$ (12).

Analysis and Isolation of Chemically Modified RNAs

RNA intermediates were isolated from reaction mixtures by spin column chromatography through Sephadex G-50 (Pharmacia) equilibrated in 100 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH 7.5 (13). RNAs were precipitated from solution by the addition of 2 vol of ethanol at -80°C in the presence of 100 mM NaCl. Electrophoresis was carried out on 1 mm thick 6% polyacrylamide gels cast and run in 90 mM Tris-borate, pH 8.3, 1 mM EDTA. Denaturing gels also contained 7 M urea. Prior to electrophoresis on denaturing gels, RNAs were melted by heating for 1 minute at 90°C in 3 M urea and immediately chilled. Autoradiographs of gels were obtained by exposure to Kodak X-Omat AR film at -80°C, with or without a DuPont Cronex Lightning Plus intensifying screen. RNAs were eluted from gels with 500 mM ammonium acetate, pH 7.5, 1 mM EDTA (13).

Dephosphorylation of 5'-pppMDV-1 (+) RNA

The 5'-terminal triphosphate groups were removed from 2 µg of MDV-1 (+) RNA by incubation with 0.7 EU calf intestine alkaline phosphatase for 30 minutes at 50°C in 50 µl 50 mM Tris-HCl, pH 8, 100 µM EDTA. Another 0.7 EU

calf intestine alkaline phosphatase was added and the incubation was continued for an additional 30 minutes. The reaction was terminated by bringing the incubation mixture to 100 mM NaCl and 0.5% sodium dodecyl sulphate and heating it for 15 minutes at 68°C. The solution was then deproteinized by extracting it twice with phenol:chloroform (1:1) and twice with chloroform (13). The dephosphorylated MDV-1 RNA was then isolated by precipitation with ethanol.

5'-^{32}P Phosphorylation of MDV-1 RNA

2 µg of dephosphorylated MDV-1 RNA were incubated for 3 minutes at 50°C in 20 µl 10 mM Tris-HCl, pH 7.5, 1 mM spermidine, 100 µM EDTA, and were then chilled rapidly. The volume was then brought to 40 µl with the addition of 12.2 pmol [γ-^{32}P]ATP (300 Ci/mmol), 30 EU T4 polynucleotide kinase, and buffer to a final concentration of 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris-HCl, pH 7.5. This solution was incubated for 75 minutes at 37°C and the reaction was stopped by bringing the mixture to 20 mM EDTA. The kinase was extracted with an equal volume of phenol:chloroform (1:1) and the phosphorylated MDV-1 RNA was isolated by precipitation with ethanol. The RNA was purified further by spin column chromatography through Sephadex G-50, followed by reprecipitation with ethanol, and then it was suspended in 100 µM EDTA-NaOH, pH 8.

Conversion of MDV-1 RNA to 5'-cystamine-MDV-1 RNA

2 µg of [5'-^{32}P]MDV-1 RNA and 16 µg of highly polymerized yeast RNA (previously dephosphorylated by incubating 180 µg yeast RNA with 50 EU calf intestine alkaline phosphatase for 1 hour at 50°C) were incubated for 3 minutes at 50°C in 20 µl of 1 mM EDTA-NaOH, pH 8, and then immediately chilled. 2.5 µl of 1 M imidazole, pH 6, and 2.5 µl of 1.5 M 1-ethyl-3,3-dimethylaminopropyl carbodiimide were then added and the mixture was incubated for 1 hour at 23°C. RNA was isolated from this mixture by spin column chromatography through Sephadex G-50. The 5'-imidazolide of [5'-^{32}P]MDV-1 RNA (together with unconverted [5'-^{32}P]MDV-1 RNA) was collected in 100 µl of 100 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7.5. 1 M 2,2'-dithiobis(ethylamine)dihydrochloride (cystamine dihydrochloride), pH 7.7, was then added to a final concentration of 250 mM, and the solution was incubated for 1 hour at 50°C. The RNA was then isolated by spin column chromatography through Sephadex G-50 and precipitated with ethanol.

Conversion of 5'-cystamine-MDV-1 RNA to 5'-biotinylated Cystamine-MDV-1 RNA

The 5'-cystamine-[^{32}P]MDV-1 RNA (together with unconverted [5'-^{32}P]MDV-1 RNA) was dissolved in 40 µl of 200 mM HEPES, pH 7.7, 1 mM EDTA, containing 360 µg N-hydroxysuccinimidobiotin, and incubated for 40 minutes at 23°C. Excess N-hydroxysuccinimidobiotin was removed by centrifugation, and the RNA was

then precipitated with ethanol. The 5'-biotinylated MDV-1 RNA (together with nonbiotinylated RNA) was further freed of excess N-hydroxysuccinimidobiotin by electrophoresis on a denaturing gel. The RNA in the ^{32}P-labeled band was eluted from the gel and purified by several precipitations with ethanol.

Separation of 5'-biotinylated MDV-1 RNA from Nonbiotinylated MDV-1 RNA

50 to 200 ng of partially 5'-biotinylated [^{32}P]MDV-1 RNA were incubated with 2 to 10 µg avidin DN for 45 minutes at 23°C in 10 mM HEPES, pH 7.7, 1 mM EDTA. The 5'-biotinylated [^{32}P]MDV-1 RNA-avidin adduct was separated from non-biotinylated [^{32}P]RNAs and free avidin by electrophoresis on a denaturing gel. The 5'-biotinylated MDV-1 RNA-avidin adduct was then eluted from the gel and precipitated with ethanol.

Identification of the RNA-biotin-avidin Adduct by its Formation of a Complex with Biotinylated Agarose

The 5'-biotinylated [^{32}P]MDV-1 RNA-avidin adduct and the 5'-cystamine-[^{32}P]MDV-1 RNA (from the control experiment shown in Fig. 3b) were eluted from the gel and precipitated several times with ethanol. Each adduct was incubated with 50 µl biotinylated agarose for 15 minutes at 23°C in 10 mM HEPES, pH 7.7, 1 mM EDTA. The agarose was precipitated by centrifugation and washed twice with buffer. The fraction of each radioactive adduct that was precipitated by the biotinylated agarose was determined in a scintillation counter.

Release of 5'-thio-ethylamino-MDV-1 RNA from the RNA-biotin-avidin Adduct

60 ng of 5'-biotinylated [^{32}P]MDV-1 RNA-avidin adduct were incubated for 1 hour at 23°C in 30 µl of 100 mM dithiothreitol, 1 mM EDTA, 10 mM HEPES, pH 7.7, to cleave the disulfide bond of the linker arm. 40 µl of biotinylated agarose were then added to the reaction mixture and incubated for 1 hour at 23°C to bind the released biotin-avidin by-product. The biotinylated agarose was removed from the solution by centrifugation. The 5'-thio-ethylamino-MDV-1 RNA was then isolated from the supernatant by precipitation with ethanol.

Kinetic Analysis of Replication

5 ng of each modified [^{32}P]MDV-1 RNA to be tested were incubated with 4.6 µg of Qβ replicase at 37°C in 50 µl of 400 µM ATP, 400 µM CTP, 400 µM [α-^{32}P]GTP (500 mCi/mmol), 400 µM UTP, 12 mM $MgCl_2$, 84 mM Tris-HCl, pH 7.5. 2 µl samples were taken every minute between 1 and 15 minutes. Each sample was absorbed onto a numbered semicircle (23 mm diameter) of 3MM filter paper (Whatman) and immersed in a beaker containing ice-cold 300 mM phosphoric acid, 20 mM sodium pyrophosphate, 1 mM EDTA. The filter papers were washed 6 times with the same solution to elute the labeled precursors from the precipitated

RNA trapped in the fibers. The filters were then washed once with ethanol, dried in air, and their radioactivity was determined in a scintillation counter.

Nucleotide Sequence Analysis

The 5'-biotinylated MDV-1 RNA-avidin adduct was used as a template for Qβ replicase under the same conditions that were described above. The [^{32}P]RNA product was deproteinized by phenol extraction, freed of labeled precursors by gel filtration chromatography through Sephadex G-50 run in 400 mM NaCl, 3 mM EDTA, 20 mM Tris-HCl, pH 7.5, and precipitated with ethanol. The MDV-1 (+) RNA was separated from the MDV-1 (-) RNA by acrylamide gel electrophoresis in the presence of 1 mM $MgCl_2$. The MDV-1 (+) RNA was then digested with ribonuclease T_1 and analyzed by two-dimensional, high-voltage electrophoresis (14) to see whether the product RNA contained the two unique oligonucleotides that are characteristic of the mutant RNA employed in these studies (6).

RESULTS

Attachment of Biotin and Avidin to the 5' End of MDV-1 (+) RNA

5'-pppMDV-1 (+) RNA, obtained by enzymatic synthesis, was converted to a 5'-biotinylated MDV-1 (+) RNA-avidin adduct in a series of steps: 1) the 5'-terminal triphosphate group was removed by incubation with calf intestine alkaline phosphatase and replaced with a 5'-terminal monophosphate by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase; 2) a 5'-phosphoroimidazole group was formed by incubation with imidazole in the presence of 1-ethyl-3,3-dimethylaminopropyl carbodiimide; 3) the imidazole group was replaced with a cystamine group by incubation with cystamine dihydrochloride; 4) biotin was linked to the cystamine by incubation with the biotinylating agent, N-hydroxysuccinimidobiotin; 5) avidin DN was bound to the 5'-biotin group; and 6) the 5'-biotinylated MDV-1 RNA-avidin adduct was separated from partially reacted intermediates by acrylamide gel electrophoresis. Figure 2 summarizes the steps required to synthesize the RNA-biotin-avidin adduct.

The electrophoretic mobility of the RNA-biotin-avidin adduct was lower than the mobility of the 5'-biotinylated RNA precursor, due to its larger size (Fig. 3a). The amount of RNA-biotin-avidin adduct formed (determined by measuring the radioactivity in each gel band) increased asymptotically as a function of avidin concentration, reaching a maximum at 25-35% adduct formed (Fig. 3b). The identity of the RNA-biotin-avidin adduct was established by three observations: 1) When 5'-cystamine MDV-1 RNA was incubated with avidin, no new slower-migrating product was seen (Fig. 3c). This demonstrates that

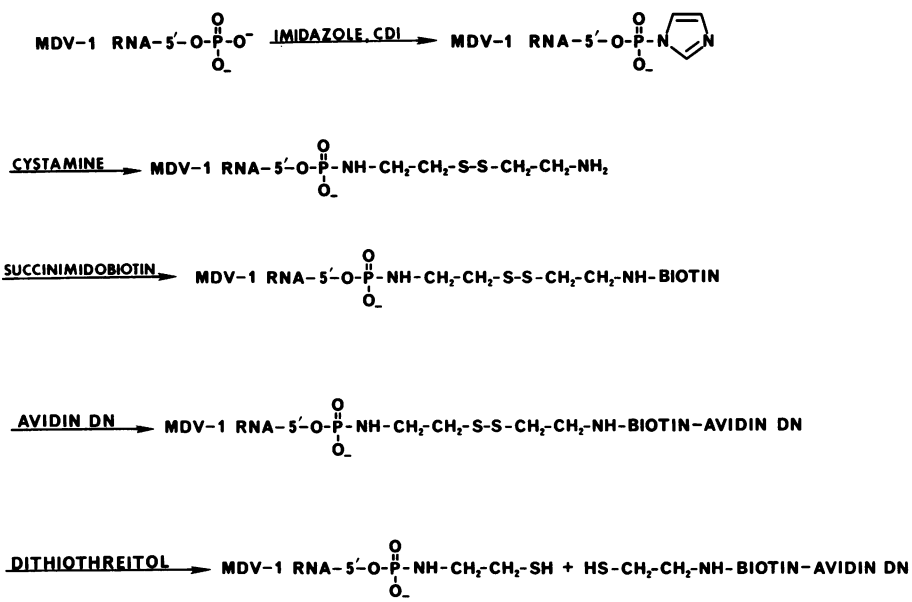


Figure 2: Synthesis of the 5'-biotinylated MDV-1 RNA-avidin adduct. Incubation of the RNA-biotin-avidin adduct with dithiothreitol cleaves the disulfide link, releasing 5'-thio-ethylamino-MDV-1 RNA.

the formation of the material in the slower-migrating band depends on the biotinylation of 5'-cystamine-MDV-1 RNA. 2) When 5'-phosphorylated MDV-1 RNA was reacted with the biotinylating agent, purified by electrophoresis, and then incubated with avidin, no new slower-migrating adduct was formed. This demonstrates that the formation of the material in the slower-moving band depends on the biotinylation of the cystamine linker. 3) More than 80% of the material eluted from the slower-migrating band bound to biotinylated agarose, while less than 10% of a control containing 5'-cystamine-MDV-1 RNA was bound. This demonstrates that the slower-migrating adduct contains avidin.

Cleavage of the Disulfide Bond Linking the Avidin-biotin Adduct to the RNA

The [^{32}P]MDV-1 RNA-biotin-avidin adduct was converted to the 5'-thioethylamino-[^{32}P]MDV-1 RNA and a biotin-avidin by-product by reduction with dithiothreitol (Fig. 2). The reduction products were incubated with biotinylated agarose to bind the biotin-avidin by-product, which was then precipitated by centrifugation. 95% of the radioactivity was recovered from the supernatant, confirming that the disulfide linker arm had been cleaved. In a control reaction, in which unreduced RNA-biotin-avidin adduct was incubated with biotinyl-

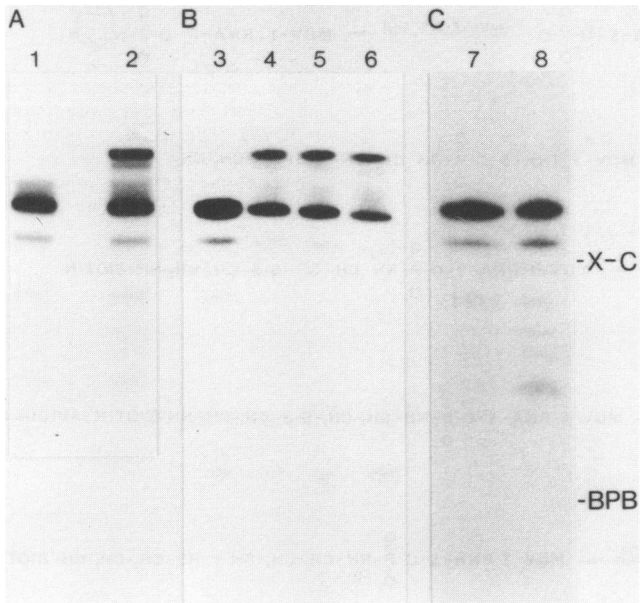


Figure 3: Electrophoretic identification of the 5'-biotinylated MDV-1 RNA-avidin adduct on denaturing gels. (a) 100 ng 5'-biotinylated MDV-1 RNA (and nonbiotinylated precursors) before reaction with avidin (lane 1) and after reaction with 10 µg avidin (lane 2). Excess avidin could be detected close to the origin by staining the gel with Coumassie blue. (b) Effect of increasing avidin concentration on formation of the RNA-biotin-avidin adduct: 1 ng 5'-biotinylated MDV-1 RNA (and nonbiotinylated precursors) before reaction with avidin (lane 3), after reaction with 1 µg avidin (lane 4), after reaction with 2 µg avidin (lane 5), and after reaction with 3 µg avidin (lane 6). (c) 1 ng 5'-cystamine-MDV-1 RNA (and precursors) before reaction with avidin (lane 7) and after reaction with 4 µg avidin (lane 8).

ated agarose, less than 10% of the radioactivity was found in the supernatant. The cleaved RNA was recovered by precipitation with ethanol and analyzed by electrophoresis on a nondenaturing gel in the presence of 100 µM dithiothreitol (to prevent dimerization of the reduced RNA). The 5'-thio-ethylamino-MDV-1 RNA migrated through the gel at the same rate as a 5'-phosphorylated MDV-1 RNA control, while the unreduced adduct migrated at a slower rate (Fig. 4).

Autocatalytic Amplification of 5'-modified MDV-1 (+) RNAs

The 5'-biotinylated MDV-1 RNA-avidin adduct and the 5'-thio-ethylamino-MDV-1 RNA were tested to see whether they could serve as templates for the synthesis of MDV-1 RNA by Qβ replicase. Separate reactions were initiated with 100 ng/ml of each 5'-modified RNA and samples were taken every minute and

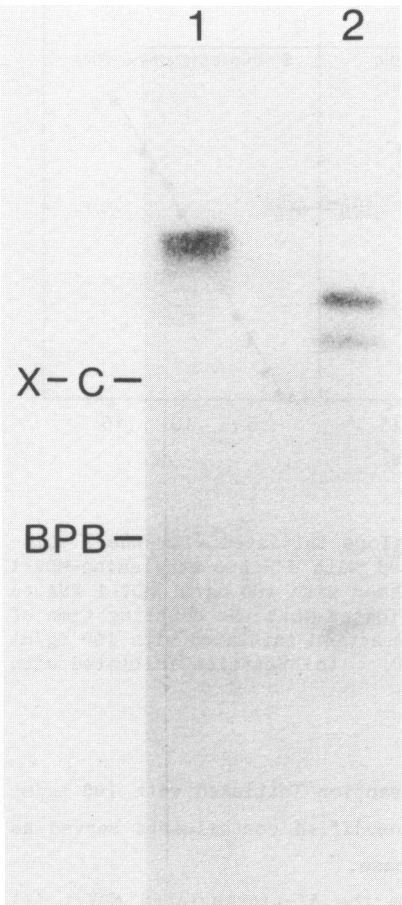


Figure 4: Electrophoretic isolation of 5'-thio-ethylamino-MDV-1 RNA on a non-denaturing gel. The 5'-thio-ethylamino derivative was generated by reduction of the disulfide link of the 5'-biotinylated MDV-1 RNA-avidin adduct: MDV-1 RNA-biotin-avidin adduct before incubation with dithiothreitol (lane 1) and after incubation with dithiothreitol (lane 2).

analyzed to determine the amount of RNA that had been synthesized. The results indicated that both of the 5'-modified RNAs served as templates for Qβ replicase (Fig. 5). The rate of RNA synthesis in each reaction was the same as the rate of synthesis seen in a control reaction initiated with 100 ng/ml MDV-1 RNA.

Reaction kinetics also indicated that the RNA that was synthesized was not due to the presence of a small amount of unmodified MDV-1 RNA in the 5'-modified MDV-1 RNA preparations. The kinetics of RNA synthesis in a control reaction initiated with only 1 ng/ml MDV-1 RNA lagged 4 minutes behind the kinetics of the reaction initiated with 100 ng/ml MDV-1 RNA (Fig. 5). This lag is the time required to make up the 100-fold difference in template concentration. Since the kinetics of the two reactions that were initiated with 5'-modified RNAs did

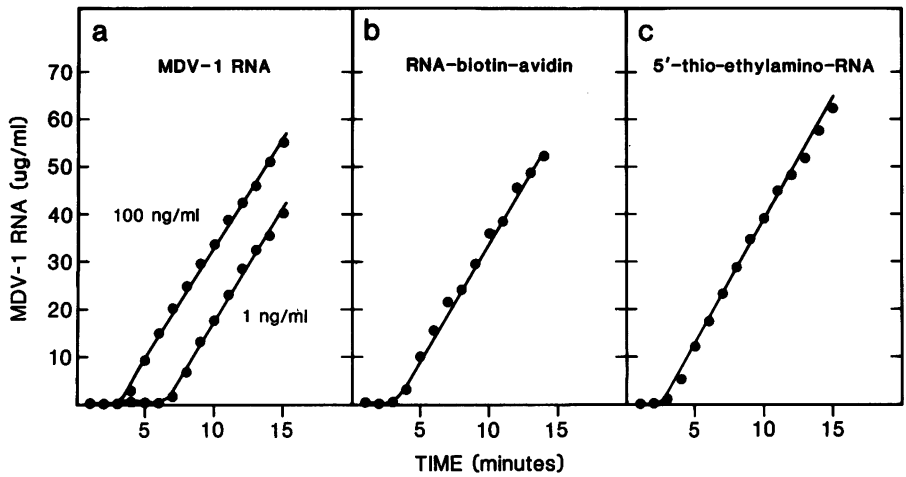


Figure 5: Kinetics of RNA synthesis in reactions initiated with the 5'-biotinylated MDV-1 RNA-avidin adduct and initiated with 5'-thio-ethylamino-MDV-1 RNA. (a) Control reactions were initiated either with 100 ng/ml MDV-1 RNA or with 1 ng/ml MDV-1 RNA. The 4 minute lag indicates that the doubling time of the MDV-1 RNA population was 36 seconds. (b) Reaction initiated with 100 ng/ml of the 5'-biotinylated MDV-1 RNA-avidin adduct. (c) Reaction initiated with 100 ng/ml 5'-thio-ethylamino-MDV-1 RNA.

not lag behind the kinetics of the control reaction initiated with 100 ng/ml MDV-1 RNA, the 5'-modified RNAs rather than unmodified contaminants served as templates for the synthesis of RNA by Qβ replicase.

Sequence Analysis of the RNA Synthesized from the 5'-biotinylated MDV-1 (+) RNA-avidin Adduct

The MDV-1 RNA used in these studies was a mutant containing three nucleotide substitutions. To confirm that the 5'-modified MDV-1 RNA served as a template, rather than a wild-type MDV-1 RNA contaminant of the replicase, the product RNA was analyzed to see whether it possessed the three mutations. RNA from a reaction initiated with the 5'-biotinylated MDV-1 RNA-avidin adduct was electrophoretically separated into its complementary strands, and the (+) strand was digested with ribonuclease T_1 and analyzed by two-dimensional, high-voltage electrophoresis (14). The resulting oligonucleotide fingerprint pattern (Fig. 6) contained two unique oligonucleotides, UAUG and UAACG, that are characteristic of the mutant MDV-1 RNA (6). This result confirms that the 5'-modified MDV-1 RNA served as the template for Qβ replicase.

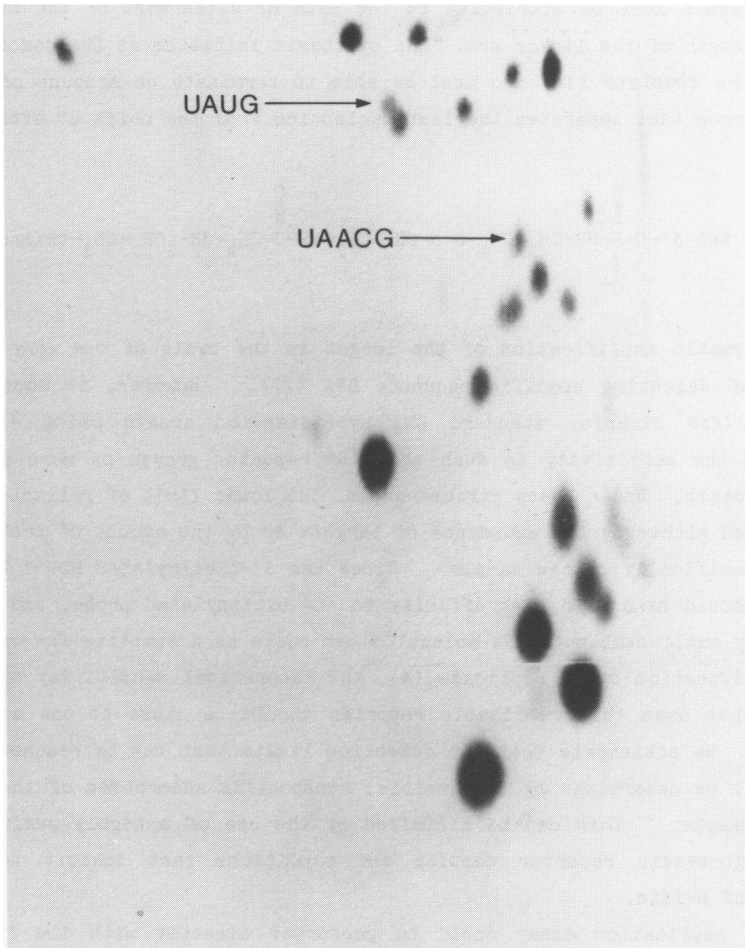
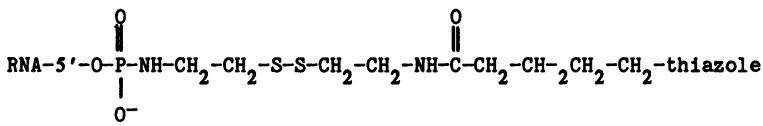


Figure 6: Fingerprint pattern of a ribonuclease T_1 digest of [α-^{32}P]GTP-labeled MDV-1 (+) RNA synthesized in a reaction initiated with the 5'-biotinylated MDV-1 RNA-avidin adduct. Arrows identify mutant oligonucleotides that are unique to the mutant MDV-1 RNA that was used in these studies.

DISCUSSION

The experiments described above show that neither the chemical procedures involved in linking biotin to MDV-1 RNA, nor the attachment of avidin to the 5'-terminal biotin group, interferes with enzymatic replication of the RNA. The first result is not surprising, since the biological activity of DNA is unaffected by treatment with a water-soluble carbodiimide at pH 6 (15). The

second result must be attributed to the site of attachment of the biotin, and to the length of the linker arm. RNA synthesis initiates at the unmodified 3'-end of the template (16) and must be able to terminate on account of the long linker group that separates the last nucleotide from the point of attachment of avidin:



Enzymatic amplification of the target is the basis of one very sensitive method of detecting specific sequence DNA (17). However, in most bioassay systems (for example, standard DNA hybridization assays using ^{32}P-labeled probes), the sensitivity is such that 10^6 reporter groups or more are needed for detection. Under these circumstances, the lower limit of reliable assay is determined either by the abundance of targets or by the amount of probe adsorbed nonspecifically to the sample. Since the 5'-biotinylated MDV-1 RNA-avidin adduct should bind with high affinity to any biotinylated probe, and since one or a very small number of RNA molecules can serve as a template for autocatalytic amplification by Qβ replicase (4), the theoretical sensitivity of an assay system that uses this replicable reporter should be close to one molecule of target. We anticipate that the detection limits that can be reached in practice will be determined by irreducible, nonspecific adsorption of the reporter to the sample. This can be minimized by the use of a highly purified MDV-1 RNA-biotin-avidin reporter complex and conditions that inhibit nonspecific binding of avidin.

The replication assay could be performed directly with the MDV-1 RNA-avidin reporter complex. For example, in nucleic acid hybridization assays, after washing away unbound reporter complexes, Qβ replicase and nucleotide precursors could be added directly to the sample. In other applications, such as the detection of an antibody bound to a cell-surface receptor, the MDV-1 RNA can be released by reduction of the disulfide linker arm before the assay. The replicated MDV-1 RNA can then be measured using either radioactive or nonradioactive methods. We are currently determining the sensitivity that can be achieved with assays of this type.

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