Volume 8 Number 19 1980 Nucleic Acids Research

Fine structure mapping of an avian tumor virus RNA by immunoelectron microscopy

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Received 20 June 1980

ABSTRACT

The RNA of a deleted strain (lacking Src gene) of an avian sarcoma virus (ASV) was examined by a newly developed immunoelectron microscopic procedure which uses anti-nucleotide antibodies as probes. After denaturation of the RNA and reaction with a high affinity, highly specific anti-7-methylguanosine-5'-phosphate (anti-pm^{7}G), 81% of 106 molecules examined were found to have antibody at one terminus, in agreement with the presence of a pm^{7}G cap in ASV-RNA. Hapten inhibition by pm^{7}G could be demonstrated. Experiments with anti-A and with anti-poly A gave results consistent with the known structure of ASV-RNA, in particular the presence of a 3' poly A tail. These studies illustrate the feasibility of using anti-nucleotide antibodies in a combined immunochemical and electron microscopic study of the fine structure of nucleic acids.

INTRODUCTION

This laboratory has been using anti-nucleotide antibodies[1] for the study of nucleic acids, chromatin and polytene and metaphase chromosomes (reviewed in ref 2). The studies on chromosomes were of an immunocytological nature and led to information on the location and organization of AT- and GC-rich clusters, as well as clusters of 5-methylcytosine. For studies on human metaphase chromosomes, procedures adaptable to the electron microscope were developed[3] and details of the arrangement of 5-methylcytosine residues near the centromeres of chromosomes 1, 9, 15, 16 and the Y could be seen.

We have now succeeded in developing procedures that permit utilization of the highly specific anti-nucleotide antibodies in electron microscopic studies of what might be called the fine structure of strands of nucleic acids. The technique is not intended as a substitute for sequencing but has attributes

of its own that should be useful in structural studies. First of all, the anti-nucleotide antibodies react only with nucleosides or nucleotides that are in single stranded regions of nucleic acids, i.e. regions in which there is no base pairing. Therefore, the antibodies can be used as direct probes of nucleic acid conformation, much as S1 nuclease is used[4]. Second, the antibodies have been shown to be highly specific. For example, antibodies to 5-methylcytosine[5], to 6-methyladenine[6] and to 7-methylguanosine-5'-phosphate[7] show only minor cross reactions with the homologous unmethylated bases. The availability of highly specific monoclonal anti-nucleotide antibodies will further enhance the value of immunoelectron microscopic procedures, and, to this end, we have recently isolated hybridomas that synthesize highly specific anti-5-methylcytosines which, by passive hemagglutination, show no reaction with cytosine. Thus, the procedure described in this paper can be a rapid reliable way of locating methylated bases along the chain of nucleic acids.

In this paper, we describe the development of procedures for the immunoelectron microscopic study of a transformation defective (td) avian sarcoma virus (ASV). The virion RNA is predominantly a 70S structure containing 2 identical subunits. The RNA of each subunit of the non-defective virus has about 9,500 nucleotides, with a 7-methylguanosine "cap" at the 5' end and a poly A tail of about 200 residues at the 3' end. It also has a number of 6-methyladenosine residues scattered throughout the genome. The strain we are examining (Bratislava 77) is transformation defective (td), and lacks an 1800 residue segment in the region of the src gene. (For a good review, see ref. 8.) We have studied the reaction of the 35S subunit with anti-7-methylguanosine-5'-phosphate (anti-pm^{7}G) of high specificity and affinity[7] and have strong evidence for the visualization of the 7-MeG "cap." Using anti-A, anti-C and anti-poly A, we have preliminary evidence for visualization of the poly A tail and A-rich regions within the RNA strand.

EXPERIMENTAL

The 35S RNA subunit of transformation defective (td) Bratislava 77 strain, an avian tumor virus (ASV), was the gift of Dr. R.V. Guntaka. It was stored on ice in 99.5% deionized formamide, 1mM EDTA, pH 7.5,

at 4 μg/ml to prevent RNase cleavage. No cleavage was detectable after 3 months of storage at 4°C.

Characteristics of the antibodies - Anti-pm^{7}G was prepared as described earlier[7]. A globulin fraction was specifically purified by adsorbing and eluting from pm^{7}G-Affigel beads as follows: A 500 μl volume of a 10% suspension of pm^{7}G-Affigel beads (Bio-Rad Affigel 703 hydrazide acrylamide beads) derivatized with pm^{7}G (P-L Biochemicals)[7] was incubated at 4°C for 24 hours on a slowly rotating pinwheel with 1 ml of the rabbit anti-pm^{7}G-BSA globulin fraction in a 1.5 ml Brinkmann polypropylene tube. The incubated beads were washed three times with 1 ml of saline, each wash followed by centrifugation. The final pellet was resuspended in 500 μl of 0.2M HCl-glycine buffer, pH 2.6, incubated 15 min at 4°C and pelleted again at 4°C. The supernatant was withdrawn, immediately neutralized with 100 μl 1M K_2HPO_4, and dialyzed in a collodion bag (Schleicher & Schuell) against saline at 4°C. The OD_{280} after dialysis was 0.37 or 270 μg of antibody per ml. This procedure normally yields about 50% of the binding activity of an equal volume of the globulin fraction, as determined by radioimmunoassay (below). RNase activity was undetectable after affinity purification as measured by TCA precipitation of ^{3}H-poly C[7].

Radioimmunoassay of anti-pm^{7}G - In 1.5 ml Brinkmann polypropylene tubes, 5 μl of affinity-purified anti-pm^{7}G was incubated with 10 μl of inhibitor and 5 μl of [^{3}H]-pm^{7}G (NEN, 5800 cpm, spec. act. 13.2 Ci/mmole, diluted in PBS) for 1.5 hours at 37°C. PBS (1 ml) was added and the solution was then filtered on a manifold with nitrocellulose filters (Gelman 0.45 μm pore size, prewet with PBS) and washed once with 1 ml of PBS. The filters were dried and counted in 10 ml Hydrofluor (National Diagnostics). About 18% of the input counts were bound by antibody without inhibitor. All assays were run in duplicate.

The purity of the inhibitors, GMP (Sigma), AMP (Sigma), pm^{7}G (P-L Biochemicals) was assessed by PEI-cellulose chromatography (PEI impregnated plastic sheets 0.1mm cellulose MN 300) developed in 0.4M NH_4HCO_3 at room temperature. One spot for each was found under short-wave UV; pm^{7}G was fluorescent.

Anti-A and anti-C were prepared[1] and specifically purified[9]. Radio-

immunoassay, Ouchterlony and tube precipitation assays showed specificity for the respective purine and pyrimidine bases. Anti-poly A was prepared according to Kahana and Erlanger[10]. It was specifically purified by R. Pohlman using a poly A-Sepharose immunoabsorbent.

Electron microscopy - A modification of the procedure of Vollenweider et al.[11,12] was used. The detergent spreading agent (BAC-Form) was stored at room temperature. It was prepared by dissolving an equal amount by weight of C_{14} and C_{16} benzyldimethylalkylammonium chloride (Fluka) at a total concentration 0.02% in 100% formamide.

Grids were coated with a 1:1 mixture of 3.5% parlodion[13] and butyl methacrylate in collodion (CAM)[14]. The CAM-parlodion grids were thinner and stronger than parlodion alone and provided better contrast in our experiments. Just before use, dried grids were floated on a 3×10^{-3}% solution of ethidium bromide in H_2O for 10 mins and then washed for 10 mins in H_2O (Szybalski, personal communication). The 10 minute exposure to ethidium bromide gave more uniform spreading consistency, despite variations of 6^{o} in temperature and 15% in relative humidity during the year. After specimens were applied (below), the grids were given a final H_2O wash of 2 mins, and the samples were dehydrated by immersion in a 90% methanol wash for 30 seconds and air-dried[15]. Methanol did not lengthen the molecules in our case but did eliminate the small discontinuities apparent after ethanol dehydration.

Grids were rotary shadowed with platinum-palladium at an angle of 7^{o} and examined in a Hitachi HU-11C transmission electron microscope at 75KV and at a magnification of about 20,000. A line grating replica (Fullam, 2160 lines /mm) was photographed with each series of micrographs, keeping the intermediate lens current constant for the series. Micrographs were projected at a 5.4X magnification. Total magnification was usually about 112,500. Molecules and antibody were traced on paper. Nucleic acid molecular lengths and positions of antibody were measured with a Numonics Graphics Calculator. In addition, ϕX174 am3CS70 single-stranded virion DNA (Biolabs) spread under the same conditions as ASV (below) was used as an external length standard; it is 5386 nucleotides long[16].

In measuring the location of antibody on nucleic acid strands, the

centers of the antibody molecules were considered the attachment site. ASV molecules and attached antibodies were measured several times and in both directions. The deviations were random averaging 0.09 cm, which corresponds to 0.008 μm at final magnification.

Preparations of specimens - A solution (100 μl) containing 10 ng of ASV, 7×10^{-4}% BAC, 56% of 99.5% deionized formamide-1mM EDTA (pH 7.5) and 44% 10mM triethanolamine (TEA)-1mM EDTA (pH 8.0) was applied as a drop to a parafilm surface in an atmosphere of at least 80% relative humidity. After 11 mins, a grid was touched to the surface of each drop. Less than 2 mins before reaction with the RNA on the grid, 1 μg of antibody (approximately 0.3 mg/ml) was diluted with 4.0 ml of 10mM TEA-1mM EDTA - pH 8.0 ("Buffer") and applied to a parafilm strip in 500 μl drops. Grids touched to ASV solutions were floated on the drops for 8 mins followed by a water wash and dehydration. The conditions for reaction with all of the antibodies were the same. For the inhibition studies, 0.25 μg of antibody was diluted with 100 μl of an 0.1% solution of the appropriate monophosphate nucleoside in "Buffer" adjusted to pH 7.5 and kept cold in ice for at least 24 hours. Immediately prior to use, it was diluted with 900 μl of "Buffer." The subsequent procedure was the same as for uninhibited antibody. Higher final concentrations of nucleotides caused aggregation of the nucleic acids; lower did not inhibit as well. Antibody-inhibitor reaction times of 1-2 hours were never as effective as 24 hours or more.

The control grids, i.e. ASV alone or ØX174 alone (external standard), were floated on 500 μl drops of "Buffer" for 8 min prior to washing with water and subsequent dehydration. No difference was observed if the buffer wash were omitted.

RESULTS AND DISCUSSION

Specificity of anti-pm^7G - The binding of the purified anti-pm^7G to pm^7G and to GMP was determined by radioimmunoassay. The results (Fig. 1) show that binding to pm^7G was with an affinity about 2000X greater than binding to GMP. AMP was even less reactive. Binding to UMP and CMP were insignificant. Since there are about 2000 GMP residues to each pm^7G residue in an ASV molecule, we can expect antibody to bind, on the average, one to two randomly

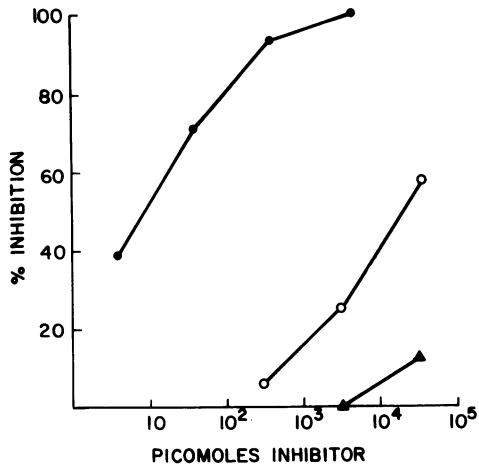

Fig. 1. Inhibition studies on anti-pm^{7}G. ●, pm ^{7}G; ○, GMP; ▲, AMP.

located places on the RNA strand in addition to the binding to the pm^{7}G cap at the 5'-end, perhaps where several GMP residues can react with the same antibody molecule and give cooperative binding. It was with this prediction in mind that we carried out and analyzed the electron microscopy studies.

Analysis of the reaction of ASV with anti-pm^{7}G - ASV-RNA, even as the 35S subunit, has considerable secondary structure, disruptable by treatment with gene 32 protein[17]. Since preliminary experiments (to be pursued further) had indicated that the pm^{7}G in the cap of native ASV-RNA might not be available for reaction with antibody and because we did not know the effect of secondary structure on the availability of other nucleotides, we sought at this time to develop procedures in which the RNA could be denatured before reaction with antibody. Conditions that denature secondary structure also will prevent antibody-RNA binding interactions. We chose, therefore, to apply the RNA to the grid under denaturing conditions and then to expose the RNA on the grid to the appropriate antibody. This procedure produced background binding of antibody to the grid, but it can be minimized by choosing the correct conditions (Experimental). The RNA, although anchored to the grid at some points, seemed not to be immobilized completely. Strand shifting and elongation appeared to be possible even while

the RNA was attached to the grid (see below).

Examination of the grids prepared in this way showed, as reported by others[18, 19] that ASV-RNA is heterogeneous in length. Only the longest strands were selected for photography and analysis. Our selection was otherwise impartial since any long, unambiguously spread molecule in a field was photographed. A histogram (Fig. 2) of 106 molecules shows a broad distribution with 89 molecules averaging 1.92 ± 0.13 μm and 17 molecules averaging 2.39 ± 0.14 μm. The former is consistent with 2.1 μm for the deleted[19] and the latter with 2.86 μm[20] for the non-deleted species. This agrees with about 16% non-deleted molecules in our preparation which, according to Guntaka (personal communication), who provided the sample, is to be expected. For the deleted species, Junghans et al.[21] reported a value of 79.7% of the intact genome; ours calculates as 80.3%, a good agreement.

If we accept the value of 2.5Å for the distance between neighboring bases[22], the non-deleted RNA calculates as 9.56 kb for the undeleted virus[19, 23]. Using ØX-174 as an external standard, we obtained values of 10.3 and 8.31 kb for the non-deleted and deleted forms, respectively.

The positions of anti-pm^{7}G on 70 RNA molecules having an antibody at one end are shown in Fig. 3A and B. In Fig. 3C are 16 undeleted molecules

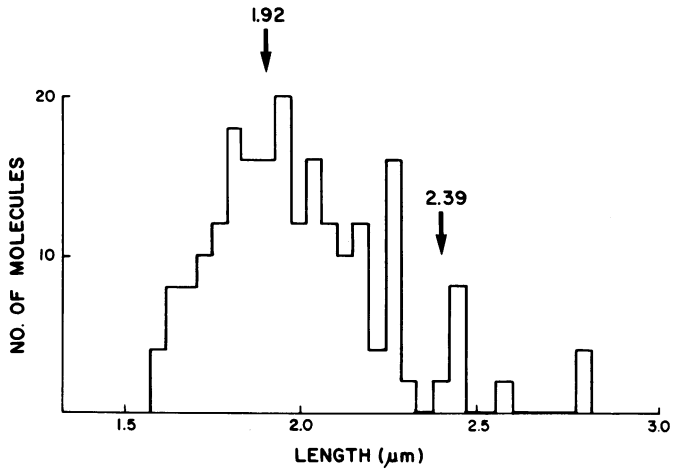

Fig. 2. Histogram showing distribution of lengths of 106 molecules of RNA.

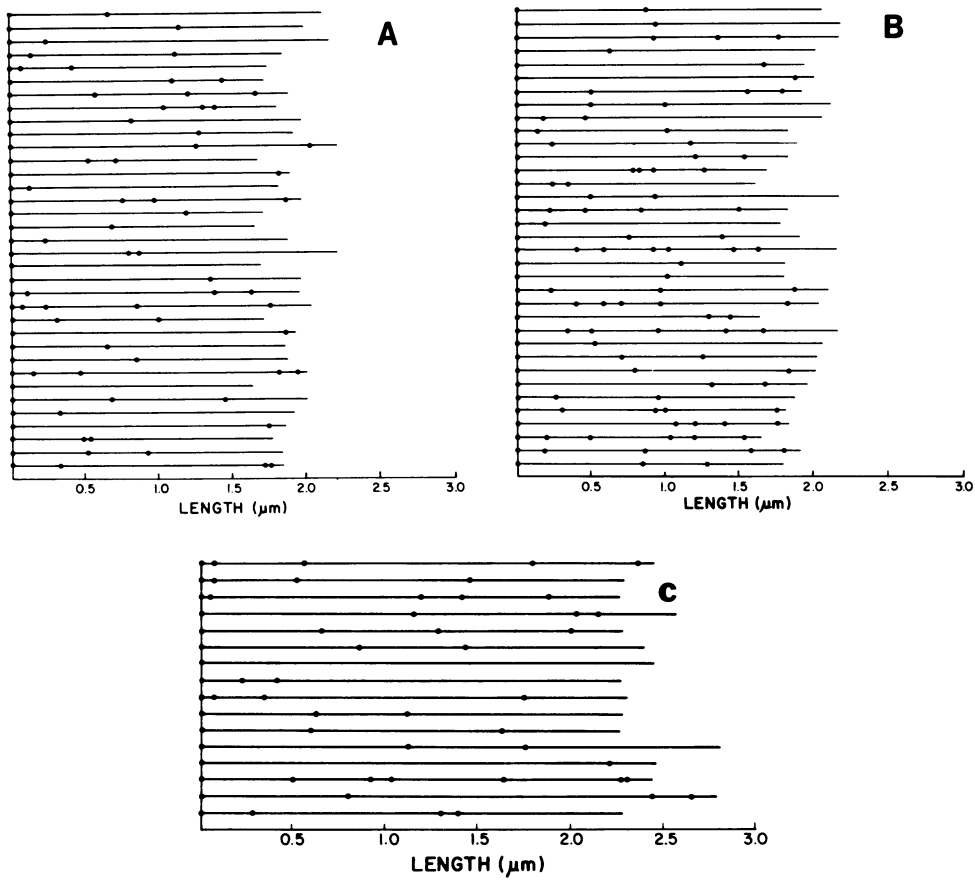

Fig. 3A, B. Positions of anti-pm^{7}G molecules on 70 deleted RNA molecules having an antibody at one terminus. C. Positions of anti-pm ^{7}G molecules on 16 undeleted RNA molecules with an antibody at one terminus.

with antibody at one end. Four molecules were seen with antibody at both ends and 16 without antibody at termini (data not shown). Of the 106, 81% had antibody at one end, 3.8% at both ends, and 15.1% had none on the ends.

Fig. 4 is a histogram of antibody distribution along 86 of the molecules with antibody at one end. All antibody molecules located within 0.09 mμ of each other were taken as being at one location. The other RNA molecules were not used in the histogram because, by inspection, it was obvious they would not affect the histogram substantially.

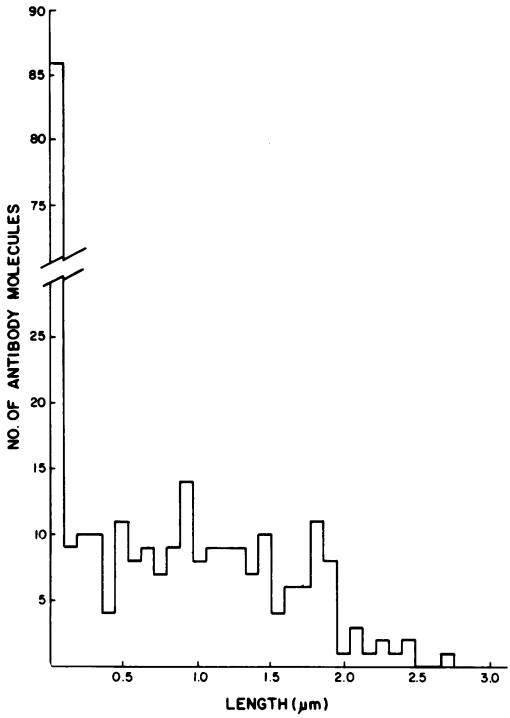

Fig. 4. Histogram of data derived from Figs. 3A, B and C, i.e. distribution of anti-pm ^{7}G molecules along strands of RNA.

The 106 molecules averaged 3.15 $\pm$ 0.99 molecules of antibody per molecules of RNA, including the molecule at the terminus. ASV is 26.7% guanine[24]. Assuming the deleted RNA has the same percentage of guanine, there is good agreement with the relative specificities of anti-pm^{7}G for pm^{7}G and GMP (Fig. 1). The histogram (Fig. 4) also shows a completely random arrangement of the antibodies that bound G residues within the RNA strand.

Shown in Fig. 5 is an example of a field spread in the absence of antibody.

Shown in Fig. 6 are some examples of fields seen in the electron microscopy of preparations of RNA treated with anti-pm^{7}G. The strands are extended to varying degrees, i.e. they have different amounts of remaining secondary structure. The strand on the upper right is highly extended; the one on the upper left still has considerable secondary structure, which can be well visualized

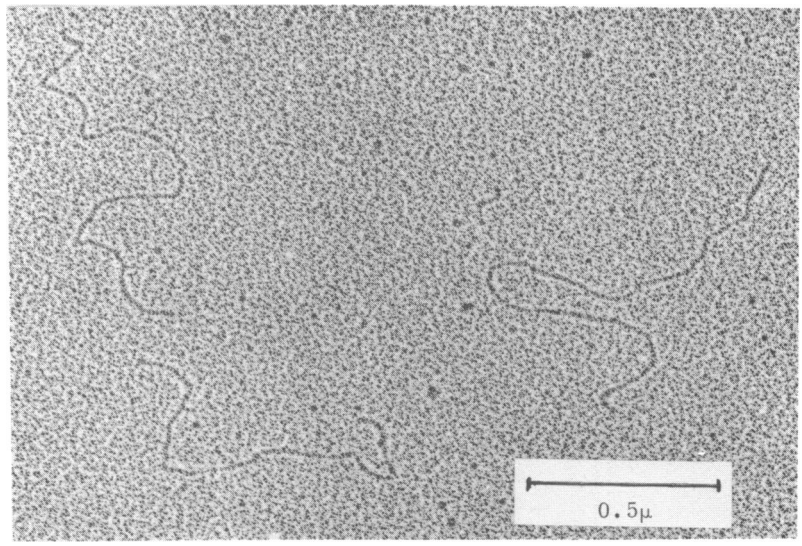

Fig. 5. ASV-RNA spread in the absence of antibody. Molecule on lower left is a fragment.

at the magnification used to measure the length of the RNA molecule (i.e. about 113,000X). All have antibody molecules at one terminus and up to three molecules bound internally.

Less than 1% of the ASV-RNA controls (i.e. RNA untreated with antibody) showed particles or secondary structure that could be mistaken for antibody and even fewer ends that were subject to question (Fig. 5).

Grids of ASV reacted with anti-pm ^{7}G, but inhibited by pm ^{7}G, contained very few molecules (less than 10%) with internal antibody and less than 5% with antibody at an end.

Studies of the reaction of ASV-RNA with other antibodies - We had shown earlier by immunochemical means[25] that anti-A could bind poly A. Grids reacted with anti-A showed many antibodies bound to ASV with especially heavy labelling at one end, presumably the poly A tail, which also appeared "kinked" as a result of reaction with antibody (Fig. 7). The distribution of the antibodies bound to the internal portions of the RNA was uneven. The RNA strands were highly extended as a result of multiple site reactions with

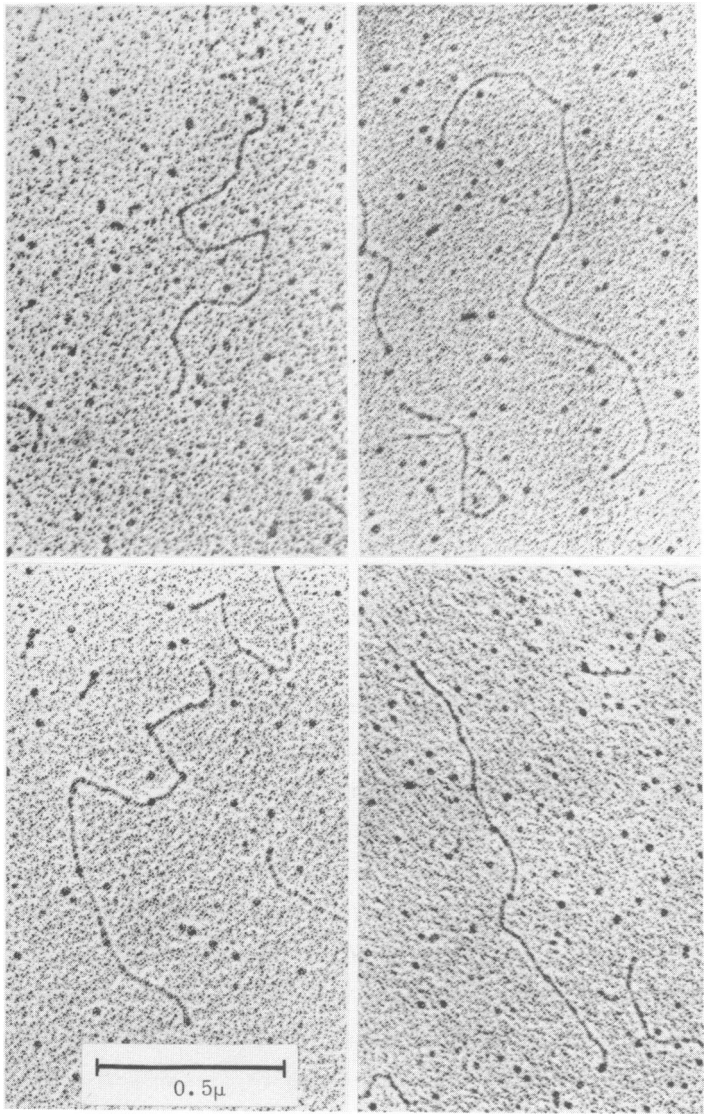

Fig. 6. Examples of ASV-RNA strands with terminal anti-pm^{7}G molecules.

antibody, much as was seen by Delius et al.[17] with gene 32 protein.

Our next studies were with anti-poly A, which had been shown to bind poly A but not AMP. A_{10} was also bound by anti-poly A but with an apparent

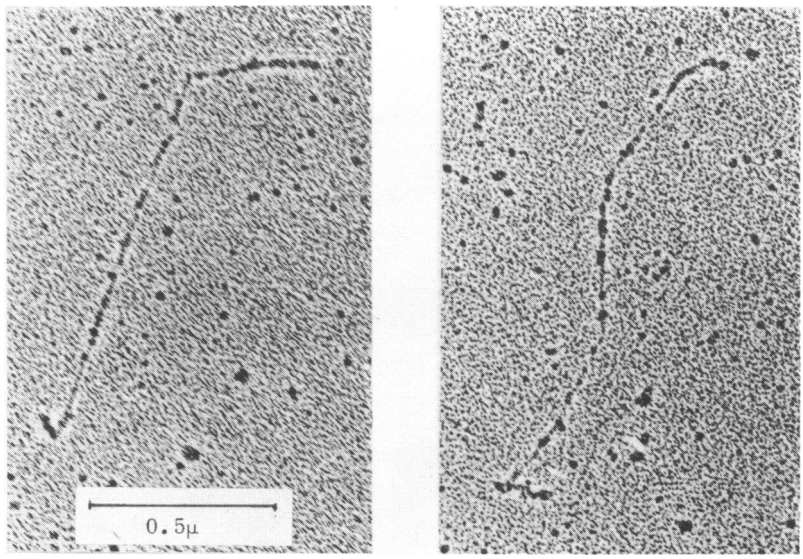

Fig. 7. Examples of ASV-RNA strands after reaction with anti-A.

binding constant several orders of magnitude less than that of poly A. In other words, its specificity was toward a conformation that could be assumed by $(A)_n$ where $n \geq 10$. Grids from reaction with anti-poly A showed 1-3 antibody molecules at one end, presumably the poly A tail and from 1-4 internal antibody molecules which appeared to be at specific sites, although a statistical study will be necessary (Fig. 8). Wang and Duesberg[26] reported preliminary evidence for A-rich runs in addition to the 3' poly A tail of 200 nucleotides, based on observed excessive binding of 11S fragments of Rous Sarcoma Virus to oligo-(dT)-cellulose. We plan to map the anti-poly A binding sites as well as the heavier binding regions of anti-A to identify poly A and/or A-rich regions of ASV, in conjunction with studies on ΦX-174, whose sequence is known[16].

Anti-C reacted with ASV-RNA, binding throughout the molecule (data not shown). Fields showed some molecules with what could be interpreted as having an unreacted poly A "tail," equivalent in size to about 300 residues. It is known (Schwartz and Gilbert, unpublished, cited in ref. 23) that C residues exist in the STR portion of the genome just preceding the poly A tail

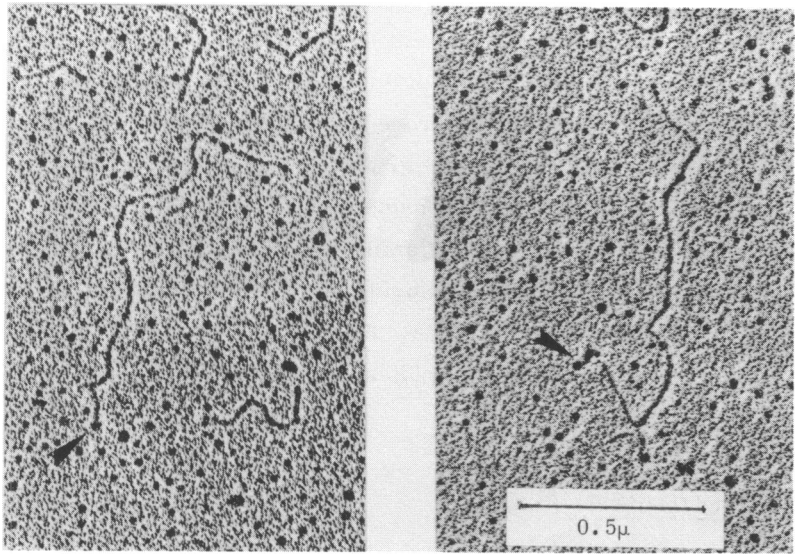

Fig. 8. Examples of ASV-RNA strands after reaction with anti-poly A. One has a single antibody molecule at one end of the RNA. The other has several. Arrowheads point to antibody.

with its approximately 200 residues. The unreacted end of the molecule seen with anti-C is somewhat longer than would be expected if the C residues in STR reacted with antibody. However, as yet, we have not examined a sufficient number of molecules. On the other hand, Junghans et al.[21] measured the poly A tail of ASV as 292 nucleotides, a discrepancy they interpreted as due to slippage of their hybrids.

One other aspect of native ASV-RNA should be mentioned: $tRNA^{trp}$ is bound to the RNA, 101 residues from the 5'-terminus of the genome[27, 28, 29]. Its interaction with the genome involves residues 2-15 as measured from its 3'-terminus[30]. Additional binding might also involve nucleotides 18-26[27]. Although we have been able to visualize samples of tRNA with the procedures used in this work (unpublished), we have not detected any on the ASV-RNA. This is to be expected considering the denaturing conditions used to prepare the 35S subunits from the 70S molecule, as well as our conditions of spreading.

The results reported above indicate that anti-nucleotide antibodies can

be used as probes to reveal information about the location of certain purine and pyrimidine bases in single-stranded regions of nucleic acids. Our data on anti-pm^{7}G are very convincing in this regard. More work has to be done with the other antibodies. In the procedure we used here, we chose to denature the RNA by spreading on the grid before reacting with antibody. This was done because ASV-RNA has considerable secondary structure[17]. Future studies will explore the reaction of antibodies with ASV-RNA before denaturation. In this way, we should get information on secondary structure and the availability of the cap in native RNA. Experiments with other nucleic acids are also in progress, as well as studies in which antibodies of other specificities are being used.

ACKNOWLEDGMENTS

These studies were funded by a National Institutes of Health grant, AI-06860 and by a grant from the National Science Foundation (NSF-PCM-77-19280). Additional support from the Union Carbide Corporation is also acknowledged. We thank R.A. Rifkind for his encouragement and advice. We would like to dedicate this paper to the late Dr. S.M. Beiser, with whom we discussed the initial plans for these experiments more than ten years ago.

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