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Intracellular Equine Arteritis Virus (EAV)-Specific RNAs Contain Common Sequences

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Equine arteritis virus (EAV) is a nonarthropod-borne togavirus. Six virus-specific RNA species have been found in EAV-infected cells having the following molecular weights: 4.3×10^6 (RNA1), 1.3×10^6 (RNA2), 0.9×10^6 (RNA3), 0.7×10^6 (RNA4), 0.3×10^6 (RNA5), and 0.2×10^6 (RNA6). RNA1 comigrates with the viral genome (M. F. Van Berlo, M. C. Horzinek, and B. A. M. Van der Zeijst, 1982, *Virology* **118,** 345–352). All RNAs hybridized with a radio-labeled cDNA probe representing RNA6, indicating that they contain common sequences. To study this homology in more detail, RNase T1 oligonucleotide fingerprinting of the RNAs was undertaken. This confirmed the presence of common sequences and showed more specifically that the intracellular viral RNAs form a nested set. The number of oligonucleotides in RNA1, however, is only one-third of the expected value. In all aspects studied the replication mechanism of EAV differs from that of other known positive-stranded RNA viruses.

Equine arteritis virus (EAV), a nonarthropod-borne member of the togavirus family (*2*) contains a single-stranded colinear infectious RNA genome with a molecular weight of about 4×10^6 (*10*). Recently, we have identified a set of five polyadenylated subgenomic RNAs in EAV-infected BHK-21 cells. The results of UV-transcription mapping experiments were consistent with a model in which the individual RNAs are derived from a larger RNA of genome size (*9*). The sum of the molecular weights of the subgenomic RNAs is 3.4×10^6 Da, which is less than the molecular weight of the genome. The information in the subgenomic RNAs could therefore be adjacent in the genome. On the other hand, the subgenomic RNAs might as well contain common sequences, as has been found, e.g., in alphaviruses, coronaviruses, and several plant viruses (*7*).

In an attempt to unravel the genetic organization of EAV, we have investigated the relationship between the EAV subgenomic RNAs. The data described in this paper show that the intracellular RNAs of EAV form a nested set.

To obtain a first impression of the sequence relationships between the EAV-specific RNAs, they were hybridized with a radio-labeled DNA probe complementary to RNA6. First, radioactively labeled total RNA, extracted from EAV-infected BHK-21 cells were separated by electrophoresis in an agarose gel and transferred to nitrocellulose paper to determine the efficiency of the blotting procedure. As shown in Fig. 1A, all the RNAs were transferred to the nitrocellulose with the exception of RNA1. Unlabeled RNAs extracted from EAV- and mock-infected cells were subsequently probed with cDNA6. The cDNA probe hybridized with RNAs 2 to 6. One additional RNA was found, however, this RNA is probably a cellular RNA since it was also present in mock-infected cells (Fig. 1A). Since RNA1 was not transferred to nitrocellulose paper, a dot blot hybridization assay with the individual purified polyadenylated RNAs of EAV was carried out (Fig. 1B). This result confirmed that the subgenomic RNAs have sequences in common and demonstrates also the virus spec-

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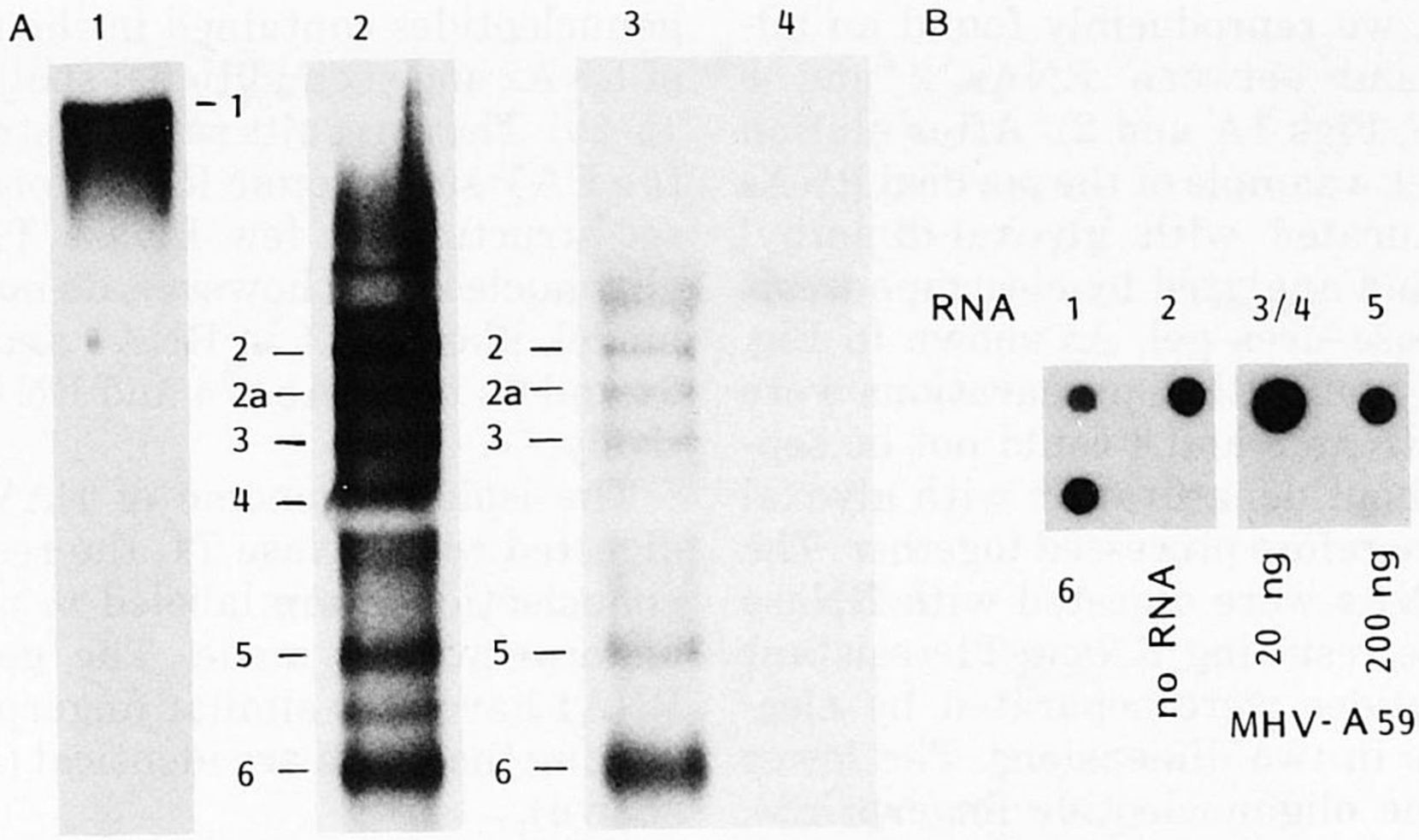

FIG. 1. Hybridization of cDNA to RNA6 with intracellular EAV RNAs. (A) Subconfluent monolayers of BHK-21 cells (approximately 4×10^6 cells/150 cm^2 tissue culture flask) were infected with EAV at a multiplicity of infection of about 30 PFU (*8*). After 1 hr adsorption the inoculum was removed and the cells were refed with phosphate-deficient medium (pH 7.2) supplemented with 5% dialyzed FCS. At 2 hr postinfection 1 μg/ml of actinomycin D was added and 1 hr later 5 mCi of ^{32}Pi was added. At 8 hr p.i. poly(A)-containing RNAs were isolated from the cells as described (*5*). The RNAs were separated by electrophoresis in formaldehyde-containing 1% agarose gels (*4*) and transferred to Gene Screen Hybridization Transfer Membrane (New England Nuclear) (lane 1: RNA remaining in the gel; lane 2: RNA transferred to nitrocellulose). Unlabeled RNAs, processed in parallel were probed by ^{32}P-cDNA to RNA6 (lane 3: infected and lane 4: uninfected cells). To prepare cDNA a 26 μl incubation mixture containing 2 μg RNA6, 0.25 μg oligo$(dT)_{12\text{-}18}$, 50 m*M* Tris-HCl pH 8.3, 50 m*M* KCl, 8 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol, 5 m*M* each of dATP, dGTP, and dTTP, 2 μCi alpha[^{32}P]CTP (3000 Ci/mmol, NEN), and 18 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wisc.) was incubated for 60 min at 42°. The reaction was stopped with 20 m*M* EDTA. RNA was hydrolyzed with 0.2 *M* NaOH for 30 min at 65°. The mixture was neutralized with HCl and buffered with HEPES-KOH, pH 7.4 (30 m*M*). Quantities of 25 μl of this mixture (about 5×10^6 cpm) were directly used as a probe. Prehybridization was for 2 hr at 43° in 5 ml of 50 m*M* sodium phosphate buffer (pH 6.8), 50% deionized formamide, 1× Denhardt's solution, 1× SSC, denatured salmon sperm RNA and poly(A) (100 μg/ml each), 0.1% glycine, and 0.1% SDS. The probe was added and after hybridization for 16 hr at 43°, the sheets were washed three times for 15 min in 1× SSC–0.1% sarkosyl and four times for 15 min at 50° in 0.2× SSC–0.1% sarkosyl. They were then dried and autoradiographed. (B) Intracellular RNA from EAV-infected BHK-21 cells was isolated 8 hr p.i. and analyzed by electrophoresis under nondenaturating conditions in 1% agarose–6 *M* urea slab gels. The individual RNAs were excised from the gel and the RNA was eluted from the gel slices by the method of Langridge *et al.* (*3*). About 2 ng of the individual RNAs was spotted on nitrocellulose (BA85, Schleicher and Schuell) and hybridized with ^{32}P-labeled cDNA6. As a negative control, 20 and 200 ng polyadenylated mouse hepatitis virus (MHV-A59) RNA was used.

ificity of the subgenomic RNAs. As a control, cDNA6 was hybridized with a 10- and 100-fold excess of mouse hepatitis virus mRNAs; no signal was detected.

Using the cDNA probe corresponding to RNA6, homology between the various subgenomic RNAs was established. By this technique one cannot, however, decide whether the positive signals are due to small stretches or to extensive regions of homology. Therefore we compared the subgenomic EAV RNAs by RNase T1 oligonucleotide fingerprinting. ^{32}P-labeled EAV-specific RNA species were isolated from infected cells as described above. After poly(A) selection the RNAs were separated by agarose–urea gel electrophoresis. In addition to the RNA species described

earlier (*9*), we reproducibly found an additional band between RNAs 2 and 3 (termed 2a; Figs 1A and 2). After elution from the gel, a sample of the purified RNAs was denaturated with glyoxal-dimethyl sulfoxide and analyzed by electrophoresis in an agarose–urea gel. As shown in Fig. 2, relatively pure RNA preparations were obtained. RNAs 3 and 4 could not be separated without denaturation with glyoxal and were therefore processed together. The purified RNAs were digested with RNase T1, and the resulting RNase T1-resistant oligonucleotides were separated by electrophoresis in two dimensions. The lower parts of the oligonucleotide fingerprints, containing the larger unique spots with more than 15 nucleotides are shown in Fig. 3. A comparison of the fingerprints leads to the following conclusions. There are four oligonucleotides (numbers 1–4) present in RNA6, three of which are also found in RNA5; spot 4 is missing in RNA5. The oligonucleotides of RNA5 are contained in the RNA species 3 and 4 (unique spots numbers 5–10) which, in turn, are a subset in the fingerprint of RNA2 (unique spots numbers 11–14). Finally, RNA1 shows all the oligonucleotides contained in the fingerprint of RNA2 and six additional spots (numbers 15–20). These results suggest strongly that the EAV subgenomic RNAs form a nested set structure. A few RNase T1-resistant oligonucleotides, however, do not fit in this model, like spot 4 in RNA6 and the spots 6a and 6b in RNAs 3/4 and RNA2, respectively.

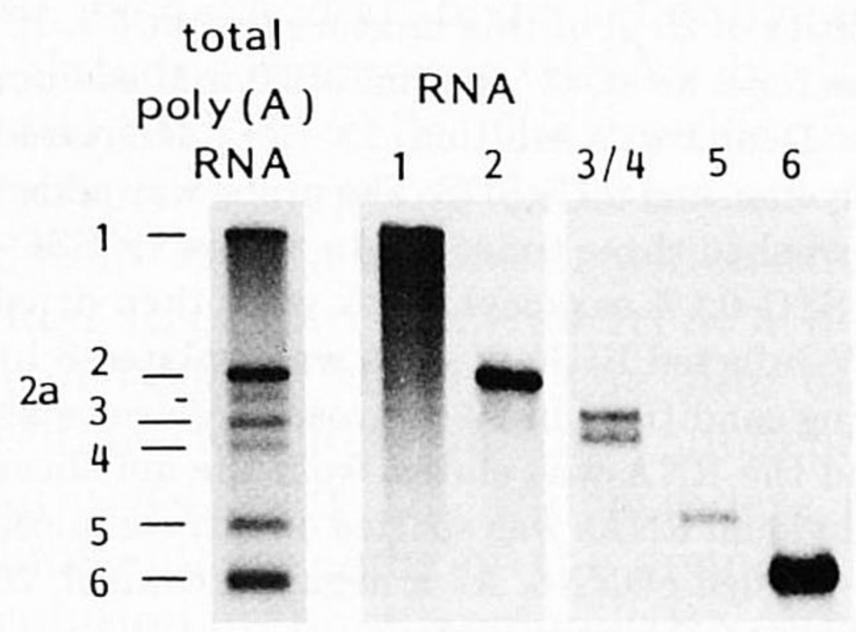

FIG. 2. Analysis of purified EAV-specific intracellular RNAs. ^{32}P-labeled intracellular RNA was prepared at 8 hr postinfection from BHK-21 cells, the poly(A)-containing fraction was isolated using oligo(dT)-cellulose chromatography and electrophoresed in an agarose/urea gel. Bands corresponding to RNAs 1, 2, 3/4, 5, and 6 were excised from the gel and RNA was eluted from the gel slices (see Fig. 1 legend). Samples of the purified fractions were reelectrophoresed after glyoxal denaturation to assess their purity, with ^{32}P-labeled unfractionated poly(A)-containing RNA serving as a marker (left lane).

The isolated genome of EAV was also digested with RNase T1, the resulting oligonucleotides were labeled *in vitro*, and a fingerprint was made. The genome and RNA1 have very similar fingerprints, suggesting that both are identical (results not shown).

Our conclusion from the experiment presented here is that the subgenomic RNAs of EAV form a nested set. The observed anomalies (spots 4, 6a, and 6b) could be the results of sequence rearrangements, during the processing of a large precursor RNA.

The experiments described here do not allow conclusions about the localization in the viral genome of the overlapping sequences. Since all the intracellular RNAs of EAV are polyadenylated it is likely that they are situated at the 3′ end of the genome. A cDNA probe complementary to the 3′ end of the EAV genome RNA should allow mapping of the common sequences; these experiments are in progress.

Fingerprinting of an RNA molecule entails complete digestion of the RNA to oligonucleotides. The numbers of RNase T1-resistant oligonucleotides can be predicted from the molecular weight of an RNA molecule (*1*). The collection of spots found in the subgenomic RNAs are roughly in agreement with the expected frequency of spots longer than 15 nucleotides. In contrast, RNA1 should have contained about three times the number of oligonucleotides of RNA2. However, instead of the expected 44 spots, only 19 spots were found. This could be due to a high G content of RNA1, but other explanations like the occurrence of polyploid sequences in RNA1 are possible.

We have shown previously by UV-transcription mapping (*9*) that the subgenomic

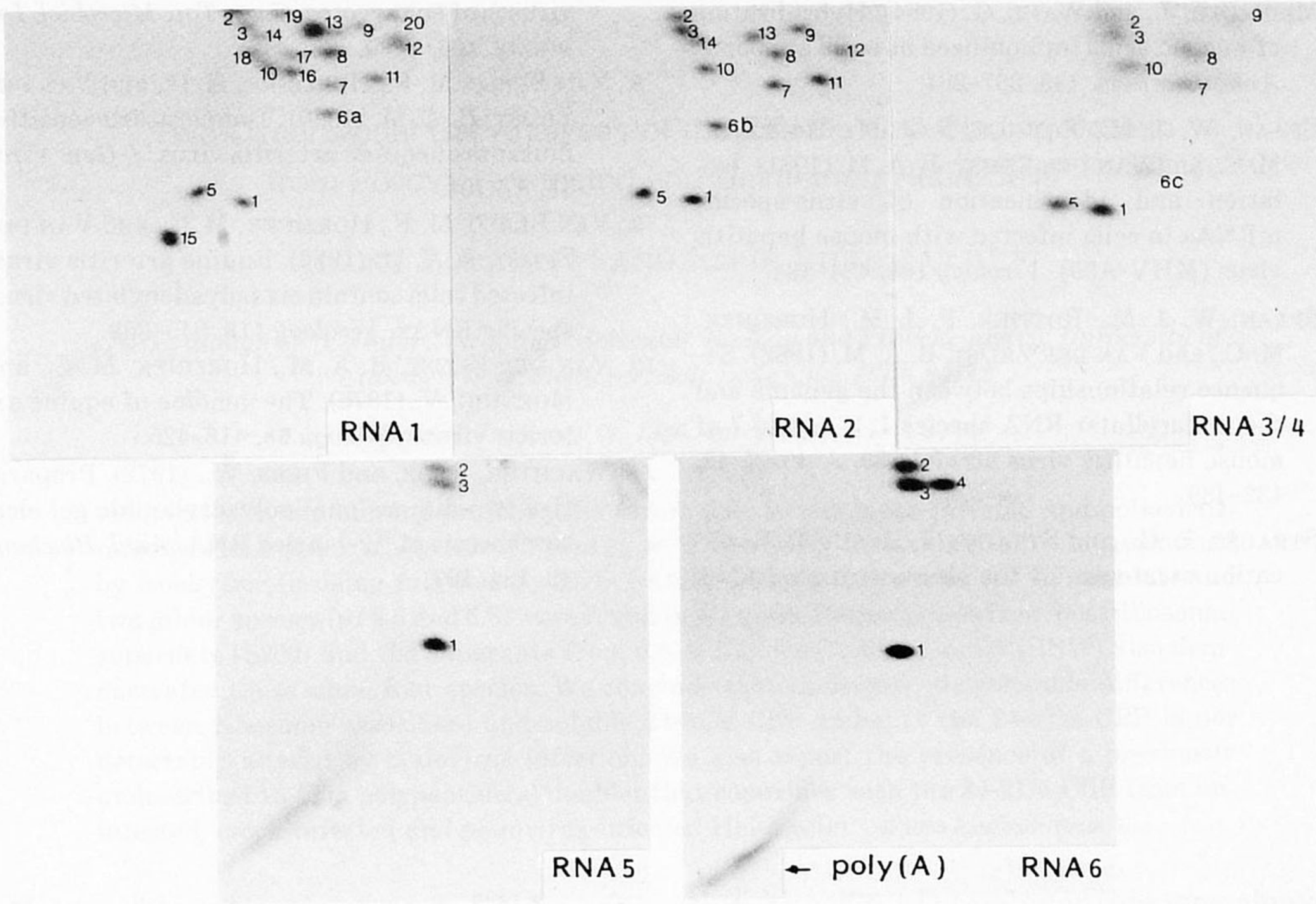

FIG. 3. RNAse T1 oligonucleotide fingerprints of the intracellular EAV RNAs. The ^{32}P-labeled intracellular RNA species were isolated as explained in Fig. 2 and digested with ribonuclease T1. The purified individual RNA species were digested with ribonuclease T1 and their oligonucleotide fingerprints obtained were electrophoresed using the method of de Wachter and Fiers (*11*), modified by Spaan *et al.* (*6*). The products were then subjected to two-dimensional gel electrophoresis and autoradiography. In these fingerprints, electrophoresis was from left to right in the first dimension and from bottom to top in the second dimension.

RNAs arise by processing of a larger precursor RNA rather than by an independent initiation of transcription. Since the EAV subgenomic RNAs form a nested set instead of being located adjacent in the genome, two different mechanisms can be postulated to explain the synthesis of these subgenomic RNAs. First, if the subgenomic RNAs are colinear with the genome, an endonuclease activity on different sites of the precursor RNA could give rise to the subgenomic RNAs, the remaining parts of the genome length precursor being degraded rapidly. Second, the subgenomic RNAs could arise by differential splicing. In the latter case but not in the first the RNAs would have a common 5′ leader sequence. It is obvious that information about the nucleotide sequences of several of the subgenomic RNAs is needed to discriminate between these possibilities.

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