JOURNAL OF VIROLOGY, Apr. 1975, p. 994-1003

Vol. 15, No. 4
Printed in U.S.A.

Nucleotide Sequence Complexities, Molecular Weights, and Poly(A) Content of the Vesicular Stomatitis Virus mRNA Species

JOHN K. ROSE* AND DAVID KNIPE

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received for publication 23 October 1974

Poly(A)-containing vesicular stomatitis virus mRNA species synthesized in vesicular stomatitis virus-infected cells have been separated into four bands by electrophoresis on formamide-polyacrylamide gels. Two-dimensional fingerprints of ribonuclease T1 and ribonuclease A digests of the RNA from each band show that they contain unique oligonucleotide sequences as well as 60 to 125 nucleotides of poly(A). The fingerprints were used to determine the nucleotide sequence complexities of RNA from three of the bands. Two contain nucleotide sequences which account completely for their molecular weights (0.70×10^6 and 0.55×10^6) determined by gel electrophoresis and sedimentation rate, and, therefore, these are radiochemically pure RNA species. The most rapidly migrating band must contain two or three different RNA species since it has a molecular weight of 0.28×10^6, determined by physical methods, and a nucleotide sequence complexity two to three times that expected for a pure RNA species of this size. These data are in complete accord with translational studies (accompanying paper) which show that each of the two pure RNA species codes for a distinct viral protein, whereas the third codes for two viral proteins. From the molecular weight and sequence complexity determinations on mRNA from the bands, we conclude that most of the vesicular stomatitis virus genome is transcribed into discrete mRNA species.

Two vesicular stomatitis virus (VSV) mRNA size classes (28*S* and 13 to 15*S*) have been resolved previously by centrifugation on sucrose gradients (5). The 13 to 15*S* species have been resolved further into three species by electrophoresis on polyacrylamide gels, whereas the 28*S* RNA is not resolved further (M. Stampfer, Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, 1972; D. Baltimore, T. Morrison, M. Stampfer, and H. Lodish, Negative Strand Virus Meet. Abstr., 1973, in press). These mRNA species are complementary to the single strand of RNA contained within the virion (5) and contain poly(A) (4, 13).

We describe here a chemical analysis of the number of VSV mRNA species contained within three of the bands separated by Formamide-polyacrylamide gel electrophoresis. The determination is based on analysis of several large, unique oligonucleotides obtained from fingerprints of the RNA from each band. The radioactivity per nucleotide length is determined for each oligonucleotide, and this number is divided into the total radioactivity in the fingerprint to obtain an apparent chain length in nucleotides (nucleotide sequence complexity) for the mRNA species in each band. The average chain length of the poly(A) in each mRNA species is calculated in a similar manner. The reliability of this technique depends on the relative abundance of mRNAs within a band, since oligonucleotides from a minor species might not be detected and would contribute only in proportion to their abundance to the total sequence complexity. A related approach has been used previously to determine the molecular weight of the VSV virion RNA (11).

MATERIALS AND METHODS

Virus and cells. Standard B particles of VSV (Indiana serotype) were grown in Chinese hamster ovary (CHO) cells and purified as described previously (6, 14, 15, 17).

^{32}P labeling and purification of VSV mRNA. CHO cells (2×10^8 to 4×10^8) growing at 37 C were infected at a multiplicity of 3 with VSV as described by Huang et al. (5), except that the medium lacked phosphate and contained fetal calf serum which had been dialyzed for 6 h against three changes (50 volumes) of 0.9% saline. The actinomycin D concentration was 5 μg/ml, and 20 mM *N*-2-hydroxyethyl-

piperazine-*N'*-2-ethanesulfonic acid (pH 7.2) was added. Thirty minutes after infection, the cells were centrifuged and resuspended in phosphate-free medium. At 1 h following infection, 10 to 20 mCi of carrier-free ^{32}P (New England Nuclear Corp.) was added. Cells were harvested by centrifugation at 4 h postinfection, resuspended in 6 ml of RSB (0.01 M Tris [pH 7.3], 0.01 M NaCl, and 1.5 mM $MgCl_2$), and allowed to swell for 10 min at 0 C. Cells were disrupted with a Dounce homogenizer, and the nuclei were removed by centrifugation (5,000 × *g*, 5 min). Nuclei were washed with 1 ml of RSB containing 1% Nonidet P-40 (Shell Oil Co.) and 0.5% desoxycholate and recentrifuged (9). The combined supernatants were adjusted to final concentrations of 0.01 M EDTA, 0.4 M sodium acetate (pH 5.2), and 1% sodium dodecyl sulfate (SDS). Five milliliters of a solution containing 50% redistilled phenol, 49% chloroform, and 1% isoamyl alcohol was added, followed by brief mixing. The mixture was centrifuged for 10 min at 12,000 × *g* (4 C), and the aqueous layer was precipitated with 2 volumes of ethanol. After centrifugation, the RNA was dissolved in 0.4 M sodium acetate (pH 5.2) and reprecipitated with two volumes of ethanol. Usually 3×10^7 to 10^8 acid-precipitable counts per minute were recovered in 2 mg of RNA when 2×10^8 cells were labeled with 20 mCi of ^{32}P.

To obtain a quantity of VSV mRNA suitable for preparative gel electrophoresis, the RNA was dissolved in 0.5 ml of binding buffer (0.4 M NaCl, 0.01 M Tris, pH 7.4, and 0.02% SDS), applied to 0.3 g of oligo(dT)-cellulose in a disposable pipette column, and washed with 5 ml of binding buffer. The bound RNA was eluted with 1 to 2 ml of 0.1 mM EDTA (pH 7.4), precipitated with 2 volumes of ethanol, and recovered by centrifugation at 100,000 × *g* for 1 h. For preparative formamide-polyacrylamide gel electrophoresis, the pellet was dried, resuspended in 20 to 50 μl of sterile water, and lyophilized. The RNA was then dissolved in sample buffer for gel electrophoresis.

Electrophoresis in formamide-polyacrylamide gels. The method described by Duesberg and Vogt (3) was used with the modification that an E.C. vertical slab gel apparatus (E. C. Apparatus Co.) was used instead of cylindrical gels. Either the four or eight slot well former was used, and wells were filled with formamide followed by underlayering of the RNA sample dissolved in formamide-glycerol as described (3). Electrophoresis at room temperature was for 16 to 20 h at 100 V in 3.75% (wt/vol) acrylamide gels. Wet gels were covered with Saran Wrap and autoradiographed for 20 s to 1 h, depending upon the amount of radioactive RNA used.

Purification of RNA from formamide-polyacrylamide gels. Using the autoradiogram as a template, the regions of the gel containing the labeled RNA bands were excised and forced through a disposable syringe (20-gauge needle) into a centrifuge tube. A volume of elution buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.4 M sodium acetate, and 0.02% SDS) twice the gel volume was added (usually about 1 to 2 ml), and the sample was mixed vigorously for 10 s at room temperature. Large gel particles were removed by centrifugation for 20 min at 30,000 × *g* (0 C). Extraction of the gel was repeated twice, and the supernatants were pooled. Greater than 90% of the RNA is eluted by this procedure. Two volumes of ethanol were added to the pooled supernatants, and the white precipitate was recovered by centrifugation at 10,000 × *g* for 10 min (0 C). The precipitate was resuspended in binding buffer (see above) and bound to a 0.2-g oligo(dT)-cellulose column followed by a 10-ml wash with binding buffer. The RNA was eluted from the column with 1 to 2 ml of 0.1 mM EDTA (pH 7.4). Sodium acetate (pH 7.0) was added to a final concentration of 0.4 M, and the RNA was precipitated with 2 volumes of ethanol at 100,000 × *g* for 1 h (0 C). The binding to and elution from oligo(dT)-cellulose effectively purifies the RNA away from a sticky gel-like material and gives excellent recovery (>90% for the three smaller VSV RNA species) if performed on an eluted sample within a few hours after electrophoresis.

RNA fingerprinting and oligonucleotide analysis. The homochromatography method of fingerprinting employed is essentially that described by Barrell (1). Unlabeled carrier yeast RNA (40 μg) was added to each ^{32}P-labeled RNA sample prior to RNase digestion. The sample was evaporated to dryness in a vacuum desiccator and resuspended in a solution containing 200 μg of RNase T1 (Calbiochem) per ml or 200 μg of RNase A (Worthington) per ml, 10 mM Tris-hydrochloride, and 1 mM EDTA (pH 7.4). Digestion was for 30 min at 37 C. Separation in the first dimension was by electrophoresis at pH 3.5 on cellulose acetate strips (3 by 55 cm; Schleicher and Schuell) at 7000 V for 25 min. Separation in the second dimension was by homochromatography on plastic-backed thin layers (20 by 20 cm) of either DEAE-cellulose or polyethyleneimine-cellulose (Brinkman Instruments). Homomixture C (1), digested for 10 min with 1 N KOH, was used for the second dimension with both DEAE and polyethyleneimine thin layers. Autoradiography of the fingerprints was from 4 h to 5 days, depending on the amount of radioactivity used.

The polyethyleneimine plates were used only for the RNase T1 fingerprints and gave better resolution of oligonucleotides than DEAE-cellulose. Oligonucleotides were eluted and digested with 0.5 N KOH, and mononucleotides were separated by electrophoresis at pH 3.5 on Whatman 3 MM paper as described by Barrell (1).

Preparation of ^{32}P-labeled rRNA markers. *Escherichia coli* (strain W3110) 16*S* and 23*S* rRNAs were labeled with ^{32}P by the procedure of Cohen et al. (2), except that labeling was for 2 h with 1 mCi of ^{32}P, and tryptophan starvation was not imposed. Cells were lysed by freeze-thawing three times in a medium containing 0.01 M Tris (pH 7.4), 0.01 M $MgCl_2$, 0.03 mg of DNase per ml, and 0.6 mg of lysozyme per ml. Phenol extraction and ethanol precipitation were performed as described above. 18*S* and 28*S* rRNA were obtained from 2×10^7 CHO cells which had been labeled for 10 h with 2 mCi of ^{32}P in phosphate-free medium. Preparation of the RNA was as described for VSV mRNA and was carried through to the step prior to oligo(dT)-cellulose binding.

RESULTS

Separation and molecular weight determination of the VSV mRNA species. To obtain uniformly labeled VSV mRNA species, VSV-infected CHO cells were labeled with ^{32}P from 1 to 4 h after infection. Actinomycin D was present to prevent host RNA synthesis. Seventy to ninety percent of the labeled RNA obtained from these cells bound to oligo(dT) cellulose, indicating that it contained regions of poly(A). The oligo(dT)-bound RNA was then fractionated on a formamide-polyacrylamide gel into the four major bands seen in the gel autoradiograph (Fig. 1). The bands, numbered 1 to 4, were reproducible, although the relative amounts of ^{32}P in each varied in different experiments. Bands 1 and 2 generally each contained from 5 to 10% of the total radioactivity in all four bands, whereas bands 3 and 4 contained 20 to 40 and 30 to 50%, respectively. RNA from the four bands were extracted from the gel and analyzed in parallel slots of another gel (Fig. 1), showing that each is largely intact and free of contamination by other species.

The molecular weight of the mRNA in each band was estimated relative to rRNA markers analyzed in adjacent slots on slab gels. The distances migrated by these marker RNAs and the VSV RNAs are plotted versus molecular weight in Fig. 2. For 23*S*, 18*S*, and 16*S* ribosomal RNA, an approximately linear relationship between the logarithm of the molecular weight and the distance migrated is observed. However, in the higher-molecular-weight region the linear relationship does not hold, and thus molecular weight assignments in this region are likely to be inaccurate. In fact, the VSV virion RNA (about 3.5×10^6 daltons, see reference 11 for a discussion of various determinations) is not well resolved from 28*S* rRNA (1.65×10^6 daltons) on these gels.

Because of the uncertainty of molecular weight assignment from gel mobility, the RNA bands which had been eluted from the gel were sedimented on sucrose gradients to assess molecular weight by different physical criteria. The sucrose gradient profile (Fig. 3) shows that bands 2, 3, and 4 have sedimentation coefficients of approximately 18*S*, 15*S*, and 12*S*, respectively, values which are consistent with the molecular weight calculated from gel mobility (see Table 3). However, band 1, which appears to be a single species when rerun on the gel, shows a broad distribution around 28*S* on the sucrose gradients, suggesting that it may be 28*S* mRNA contaminated with other RNA species.

Thus, bands 2, 3, and 4 are the RNA species which have been designated 13 to 15*S* on sucrose gradients of VSV mRNA (5), whereas band 1 presumably contains mainly the 28*S* species which codes for the large viral protein (8).

Fingerprint analysis of the isolated RNA species. To examine the nucleotide sequence relationships among the purified RNA species, and to examine their nucleotide sequence complexities, RNase A digests of each were separated by electrophoresis and homochromatography. The autoradiographs of these fingerprints are shown in Fig. 4. Separation in the first dimension (cellulose acetate, pH 3.5) is mainly by charge, whereas the second dimension (homochromatography) separates mainly by size with the smaller oligonucleotides moving furthest toward the top of each plate (1). The sequences of the dinucleotides at the top of the figures are indicated. Extending from these dinucleotides are lines of oligonucleotides of increasing size containing additional adenylic acid residues.

The compositions of several of these oligonucleotides were determined and are indicated in the figures. Note that band 4 contains the sequences AC and AAC, but lacks larger sequences in the A_nC line, whereas in band 3 this line is complete to A_6C. Band 2 contains the sequence AC (displaced due to uneven chromatography), AAC, and A_5C, but lacks A_4C. In band 1 the line is complete only to A_4C. Band 2 also contains a characteristic oligonucleotide doublet (numbered 4 and 5) which is never seen in band 1 fingerprints. Thus, band 1 could not be a precursor of bands 2 or 3 since it lacks sequences which they contain. Sequence differences are also clear in the adjacent line A_nGC, where the band 1 and 4 fingerprints contain an oligonucleotide with the composition A_4GC, whereas bands 2 and 3 lack this oligonucleotide (position indicated by arrows in fingerprints of bands 2 and 3). The large oligonucleotides near the bottom of the plates show patterns which are characteristic of each RNA species, again indicating that the RNA bands are not precursors or products of each other.

RNase T1 fingerprints of bands 3 and 4 were also obtained for nucleotide sequence complexity analysis. These fingerprints are shown in Fig. 5 with sequences of the mono- and dinucleotides indicated. As in the RNase A fingerprints, the larger oligonucleotides from bands 3 and 4 differ significantly. Analysis of oligonucleotides from both RNase A and RNase T1 fingerprints indicated that all four nucleotides

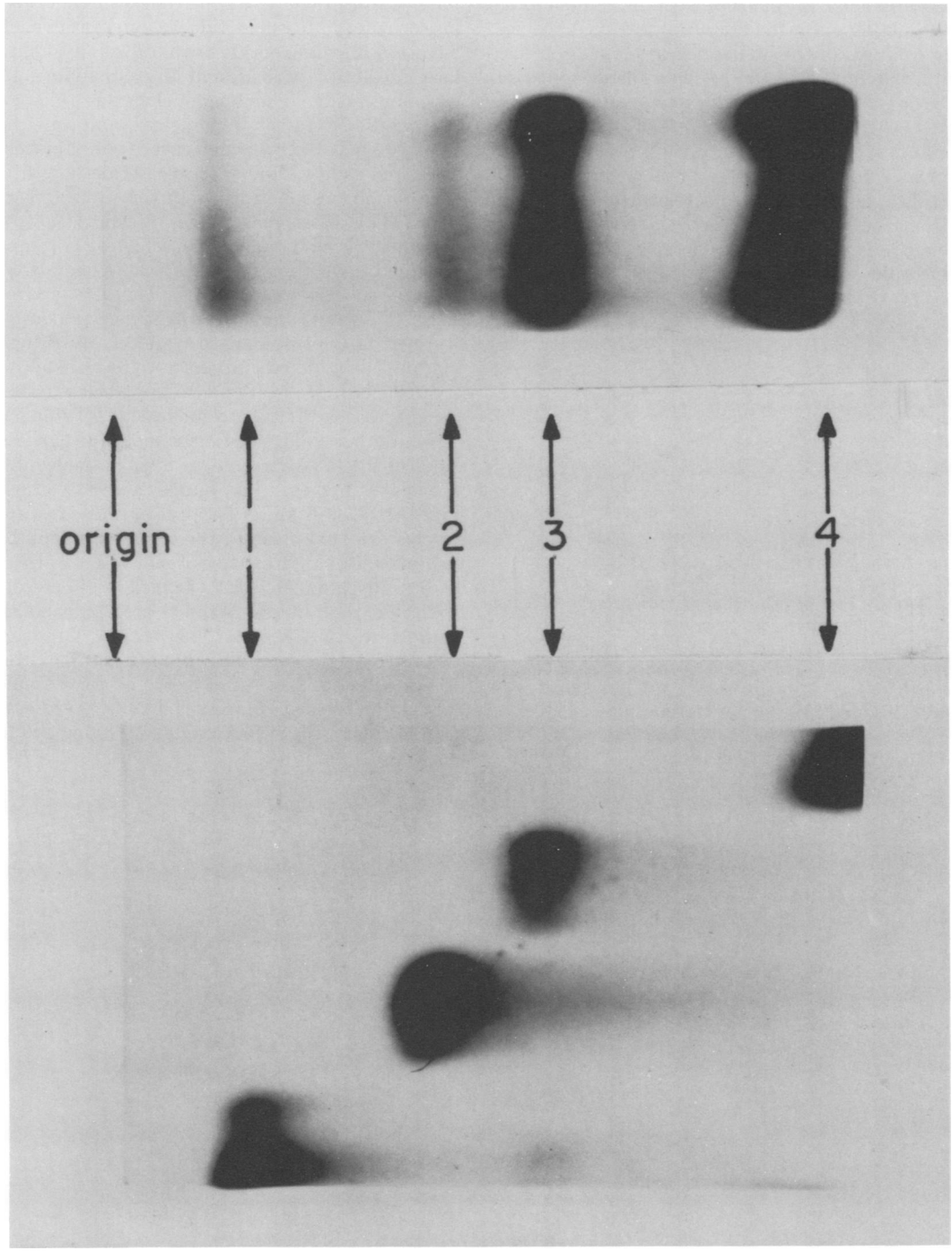

FIG. 1. *Autoradiograph of the VSV mRNA species separated on a formamide-polyacrylamide gel and electrophoresis of the isolated RNA bands. Electrophoresis was on 3.7% formamide-polyacrylamide slab gels as described in Materials and Methods.*

were labeled to the same specific radioactivity, indicating uniform labeling of the RNA species.

Analysis of the nucleotide sequence complexity of bands 2, 3, and 4 was carried out from RNase A fingerprints of band 2 and from both RNase A and T1 fingerprints of bands 3 and 4. Band 1 contained insufficient radioactivity to permit analysis. To determine complexity, several large isolated oligonucleotides were excised from the fingerprints, and the radioactivity in each was determined. The size of the oligonucleotide was determined from analysis of its base composition, assuming that a single uridylate or cytidylate nucleotide was present in each RNase A oligonucleotide or that a single guanylate nucleotide was present in each RNase T1 oligonucleotide. The oligonucleotides selected are numbered on the fingerprints, and analysis is shown in Table 1. In each case, approximately the same radioactivity per nucleotide length is seen for oligonucleotides from the same fingerprint. The most reasonable interpretation of this result is that each oligonucleotide contains a sequence which occurs once within each RNA species, and that each band contains one or more RNA molecules with the same radioactivity per nucleotide length. The analysis was completed by determining the total radioactivity in each fingerprint and dividing this number by the radioactivity per nucleotide (Table 2). This number should equal the length in nucleotides of the RNA molecule if a single molecular species is present. For bands 2 and 3, these numbers (2,156 and 1,545 nucleotides, respectively) are very close to the nucleotide lengths expected for single species of molecular weights 0.78×10^6 and 0.56×10^6 (Table 3). This result indicates that bands 2 and 3 are radiochemically pure RNA species. For band 4, however, the range of the possible sequence complexities is two to three times that expected for a single molecular species. The most likely interpretation of this result is that band 4 contains two to

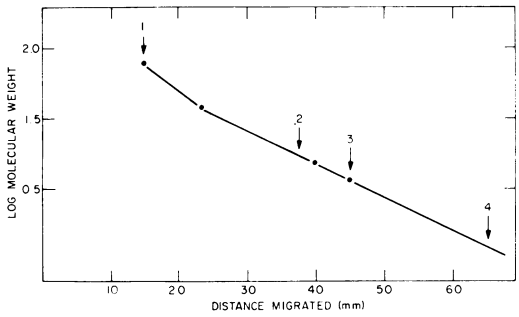

FIG. 2. *Plot of logarithm of molecular weight versus distance migrated for marker rRNAs and VSV mRNAs. The marker RNAs (positions indicated by points) were E. coli 23S and 16S rRNA, molecular weights of 1.1 × 10⁶ and 0.55 × 10⁶, respectively (16), and CHO cell 28S and 18S rRNAs, molecular weights of 1.65 × 10⁶ and 0.65 × 10⁶, respectively (10).*

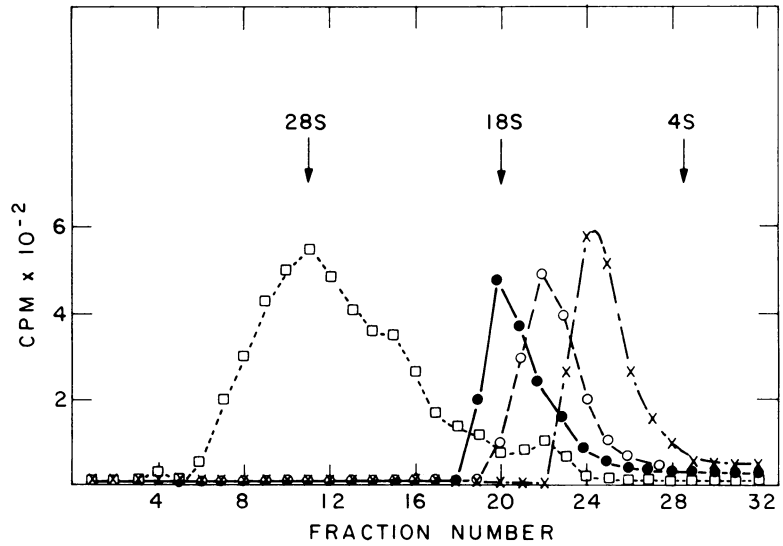

FIG. 3. *Sedimentation of isolated VSV mRNA bands on SDS-sucrose gradients. ^{32}P-labeled VSV mRNA species isolated from a formamide-polyacrylamide gel as described in Materials and Methods were sedimented on separate 15–30% linear sucrose (wt/wt) gradients (0.5% SDS, 0.1 M NaCl, 0.01 M Tris, pH 7.5, 1 mM EDTA) in an SW41 rotor for 14 h at 25,000 rpm. Positions of the CHO 28S, 18S, and 4 to 5S marker RNAs are indicated. Symbols: □, band 1 RNA; ●, band 2 RNA; ○, band 3 RNA; ×, band 4 RNA.*

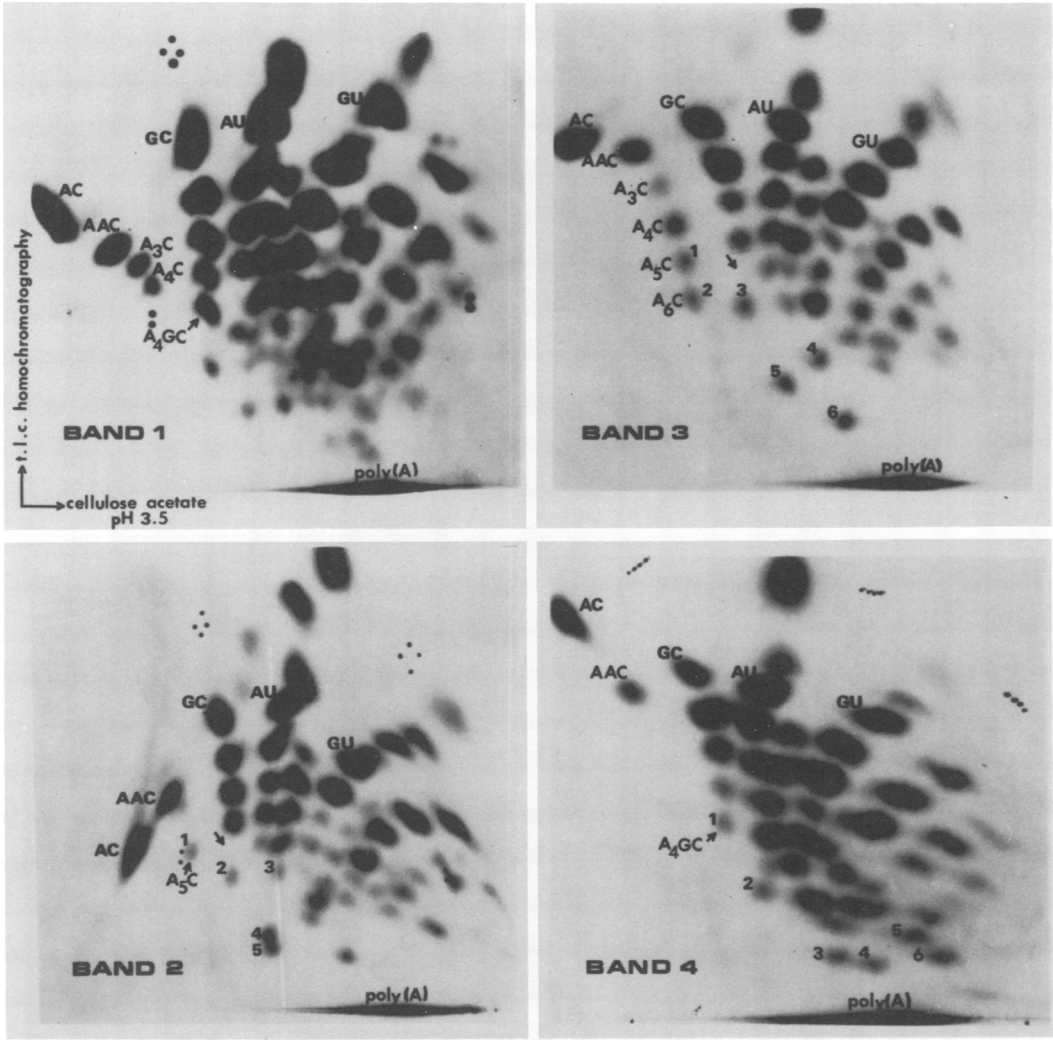

FIG. 4. *Autoradiographs of two-dimensional separations by electrophoresis and thin layer homochromatography (DEAE plates) of oligonucleotides produced by complete RNase A digestion of VSV mRNA bands 1 to 4. The spots just above and to the right of the dinucleotides AU and GU are their cyclic 2',3'-phosphates. Extensive RNase A digestion conditions which minimize these also result in digestion after A residues.*

three RNA species (see Discussion).

Size analysis of poly(A) from the fingerprints. The large oligonucleotide(s) visible at the origin of the second dimension in each of the fingerprints (Fig. 4 and 5) was eluted from the thin layer and digested with 0.5 N KOH, and the products were analyzed by pH 3.5 paper electrophoresis. The analysis showed that it was greater than 90% adenylate and therefore presumably represents the poly(A) from each messenger which has been identified in the total VSV messenger population by other methods (4, 13). Since the counts per minute per nucleotide are known for bands 2, 3, and 4, the counts per minute in the poly(A) region of the fingerprints can be used to calculate the average length of poly(A) on each messenger species. The calculation (Table 3) shows that the average poly(A) size on these species ranges from 64 to 124 nucleotides. The calculation for band 4 is made assuming that two different RNA species are present within the band, and that the poly(A) is equally distributed between them.

Since the counts per minute per nucleotide length could not be determined accurately for the band 1 fingerprint, the length of the poly(A) was calculated by assuming that the fraction of the total fingerprint radioactivity in poly(A) is

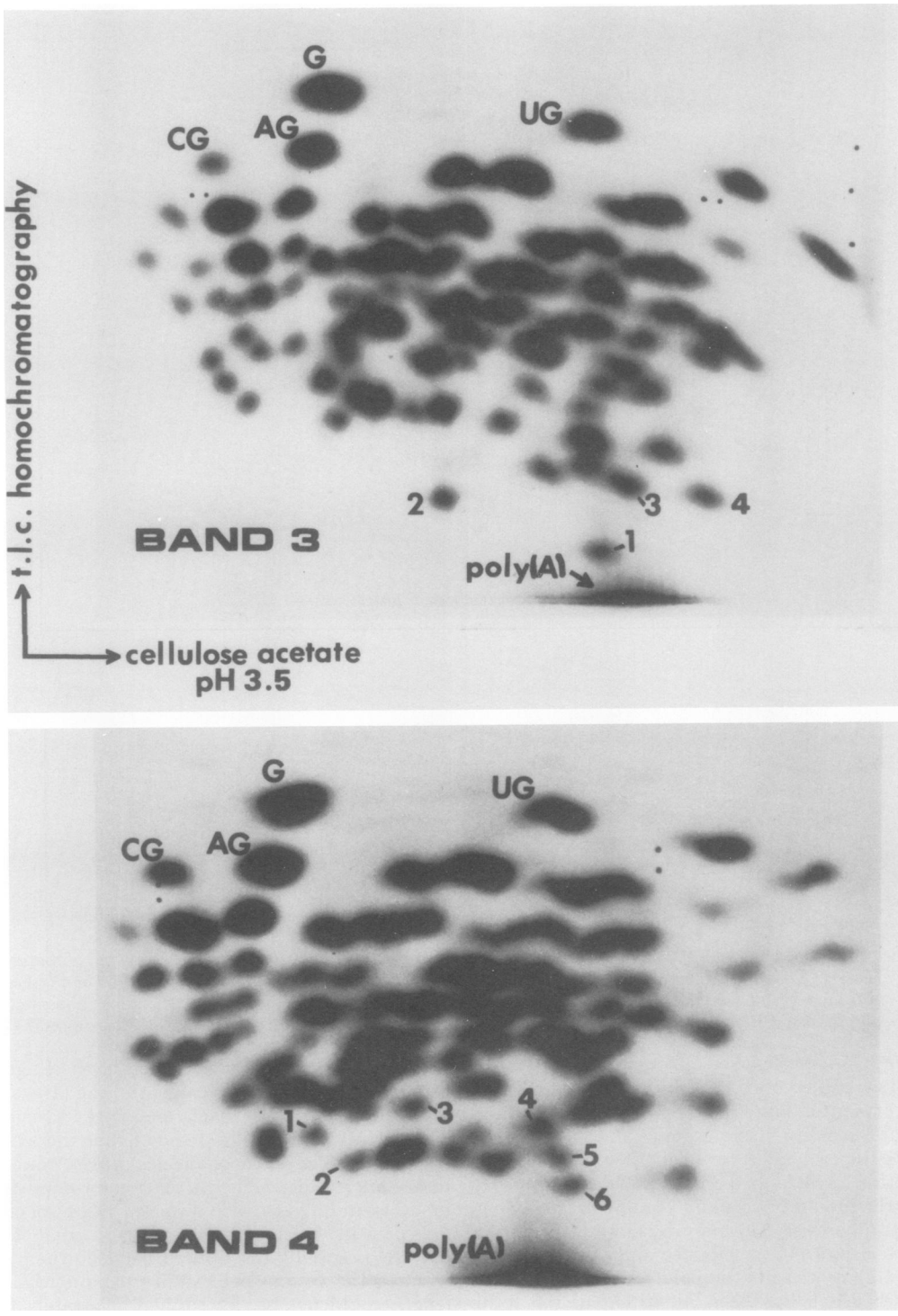

FIG. 5. *Autoradiographs of two-dimensional separations by electrophoresis and thin layer homochromatography (polyethyleneimine plates) of oligonucleotides produced by complete RNase T1 digestion of VSV mRNA bands 3 and 4.*

TABLE 1. *Analysis of oligonucleotides in RNase A and RNase T1 fingerprints of bands 2, 3, and 4*

Band no.	RNase	Oligonucleotide[a] designation	Composition	Length (nucleotides)	Counts/min	Counts/min per nucleotide
2 (Fig. 4)	A	1	A_5C	6	148	25
		2	GA_6C	7	162	23
		3	G_2A_5C	8	190	24
		4	G_2A_7C	10	200	20
		5	G_3A_7C	11	218	20
3 (Fig. 4)	A	1	A_5C	6	412	69
		2	A_6C	7	494	71
		3	GA_5C	7	542	77
		4	GA_7U	9	659	73
		5	G_2A_7C	10	748	75
		6	GA_9U	11	835	76
3 (Fig. 5)	T1	1	$C_3A_6U_4G$	14	1,548	110
		2	$C_3A_5U_2G$	11	1,248	113
		3	$C_3A_4U_2G$	10	1,051	106
		4	$C_2A_3U_4G$	10	962	96
4 (Fig. 4)	A	1	GA_4C	6	648	108
		2	G_2A_6C	9	854	95
		3	G_4A_4U	9	770	86
		4	ND[b]	10[c]	752	75
		5	ND	8[c]	810	101
		6	ND	9[c]	912	101
4 (Fig. 5)	T1	1	C_4A_3UG	9	2,984	330
		2	CA_6U_2G	10	3,423	342
		3	$C_2A_4U_2G$	9	3,414	379
		4	$C_3A_2U_4G$	10	2,972	297
		5	$C_3A_3U_4G$	11	3,563	323
		6	$C_3A_4U_4G$	12	3,844	320

[a] The oligonucleotides included in the table are those which gave minimal values of counts per minute per nucleotide length. Other oligonucleotides which gave integral multiples of these values were analyzed. For example, A_4C in the band 3 RNase A fingerprint contained 145 counts/min per nucleotide and was presumed to be present in two copies per molecule. Such multiple-copy oligonucleotides were omitted from the analysis.

[b] ND, Not determined.

[c] Estimated from position on fingerprint.

TABLE 2. *Calculation of nucleotide sequence complexities and poly(A) length for bands 2, 3, and 4*

Band no.	RNase	Counts/min per nucleotide (average or range)	Total counts/min in fingerprint[a]	Counts/min in poly(A)	Total counts per min/counts per min per nucleotide	Counts per min in poly(A)/ counts per min per nucleotide
2	A	23	49,600	2,871	2156	124
3	A	73	110,539	5,100	1514	70
3	T1	106	167,130	6,105	1576	58
4	A	75-101	210,424	15,909	2,083-2,805	170
4	T1	297-379	799,812	61,810	2,110-2,692	180

[a] Determined by excising all radioactive regions from the thin layer and counting them in a scintillation spectrometer. In RNase A fingerprints, the loss of CMP (not transferred to 2nd dimension) was corrected for.

the fractional length of the molecule which is poly(A). The major species present is assumed to be 28S with a molecular weight near 1.65×10^6 (Fig. 2 and 3), corresponding to about 4,500 nucleotides, and the fraction of the total ^{32}P in poly(A) is calculated to be a length of poly(A) of about 110 nucleotides.

Determination of average poly(A) length for

TABLE 3. *Molecular weights, nucleotide sequence complexities, poly(A) content, and coding capacities of the VSV mRNA species*

Band no.	Mol wt from gel mobility	Sedimentation coefficient	Nucleotide sequence complexity	Avg poly(A) length	Amino acid coding capacities (daltons)
1	$\geq 1.65 \times 10^6$ (4,600 nucleotides)	Heterogeneous (28*S*)	ND[a]	110	170,000[b]
2	0.70×10^6 (1,940 nucleotides)	18*S*	2,156	124	74,500[c]
3	0.55×10^6 (1,530 nucleotides)	15*S*	1,545[d]	64	54,200[c]
4	0.28×10^6 (780 nucleotides)	12*S*	2,100–2,800	90	74,000–100,000[c]

[a] ND, Not determined.
[b] Calculated from molecular weight determined by sucrose gradient sedimentation.
[c] Calculated from nucleotide sequence complexity assuming poly(A) is noncoding and an average molecular weight of 110 for amino acids.
[d] Average of RNase A and RNase T1 determinations shown in Tables 1 and 2.

the VSV mRNA species showed considerable variation in different experiments, although it was always greater than 50 nucleotides for each species. This variation may reflect uncontrolled variation in the rate of poly(A) synthesis or degradation in different preparations.

Since no distinct RNA species were found in the 10 to 30% of the RNA which did not bind to oligo(dT)-cellulose, we conclude that all VSV mRNA species analyzed contain poly(A) sequences. The average lengths of these sequences are consistent with the wide range of poly(A) sizes reported previously (4) for a mixture of VSV mRNAs.

DISCUSSION

These results show that two-dimensional RNA fingerprinting techniques can be used to assess sequence relatedness of large viral mRNA molecules as well as to examine their nucleotide sequence complexity, poly(A) content, and purity.

A major difficulty was encountered initially in attempting to determine the molecular complexities of the VSV mRNAs from fingerprints of RNase A digestions. If sufficient carrier RNA was not added during the digestion, significant digestion of the poly(A) was observed as well as loss of other large oligonucleotides, presumably due to a low level of cleavage after adenylate. This problem was not encountered with fingerprints of RNase T1 digests. Also, streaking of the RNAs during gel electrophoresis due to gel overloading must be avoided, since it results in contamination of each mRNA band with oligonucleotides from the other bands and overestimates of sequence complexities.

The validity of the sequence complexity determinations are confirmed by the agreement between complexities determined from RNase A and RNase T1 fingerprints of bands 3 and 4 and the close agreement between sequence complexity and molecular weight for bands 2 and 3. Furthermore, the RNAs extracted from bands 2 and 3 code for the viral proteins G and N, respectively (accompanying paper), and their nucleotide sequence complexities are just sufficient to encode these proteins.

The sequence complexity of band 4 RNA relative to its molecular weight suggested that it contained two to three RNA species. Analysis of band 4 RNA is complicated, however, because the large oligonucleotides analyzed are presumably derived from RNA species which may differ in length and counts per minute per nucleotide length. In fact, more variation in counts per minute per nucleotide was observed in band 4 oligonucleotides than in oligonucleotides from the other species. For this reason, only a possible range of sequence complexities is given in Table 3. That band 4 does contain at least two mRNA species is shown by its ability to direct synthesis of two VSV proteins, M and NS, which require at least twice the coding capacity of a single mRNA of the size of band 4. However, until the band 4 RNAs can be separated, their total nucleotide sequence complexity must be considered tentative.

The formamide-polyacrylamide gels separate bands 2, 3, and 4 into species which are apparently of homogeneous size when analyzed by sucrose gradient sedimentation, and their sedimentation rates are consistent with molecular weights calculated from gel mobility relative to

markers. However, band 1 RNA shows a broad sucrose gradient profile, suggesting that it may be a mixture of RNAs of different sizes which are not resolved on the gel. The band 1 fingerprint shows that it lacks nucleotide sequences found in the smaller RNAs, and thus the heterogeneity is not due to contamination with smaller RNAs or large amounts of a precursor to the smaller RNAs. We feel that the most likely explanation for the heterogeneity of band 1 is that it contains mainly the 28*S* mRNA (5, 8) as well as some large degradation products of it, and perhaps a small amount of the 40*S* (+) strand copied from the entire (−) strand VSV genome (D. Baltimore, T. Morrison, M. Stampfer, and H. Lodish, Negative Strand Virus Meet. Abstr., 1973, in press).

The total nucleotide sequence complexity of bands 2, 3, and 4 is about 4,700 [without poly(A)], and the 28*S* L protein messenger (8), which is presumably contained within band 1, must contain about 4,400 nucleotides (see above). Thus, the total nucleotide sequence complexity of the five monocistronic mRNAs coding for the five viral proteins must be about 9,110, corresponding to a total molecular weight of 3.2×10^6. Thus, the nucleotide sequence complexities of the VSV mRNAs account almost entirely for the 3.5×10^6 molecular weight of the (−) strand virion RNA.

ACKNOWLEDGMENTS

We thank Craig Squires for patient instruction in nucleotide sequencing techniques and Harvey F. Lodish and David Baltimore for helpful discussions of the work. We are especially grateful to Alan Jacobson for instruction in the use of formamide-polyacrylamide gels.

This work was supported by American Cancer Society grant no. VC-4D and Public Health Service grant AI-08814 from the National Institute of Allergy and Infectious Diseases. J.K.R. is a postdoctoral fellow of the National Cystic Fibrosis Research Foundation, and D.K. is a predoctoral fellow of the National Science Foundation.

LITERATURE CITED

1. Barrell, B. G. 1971. Fractionation and sequence analysis of radioactive nucleotides, p. 751-828. G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research, vol. II. Harper and Row, New York.
2. Cohen, P. T., M. Yaniv, and C. Yanofsky. 1973. Nucleotide sequences from messenger RNA transcribed from the operator-proximal portion of the tryptophan operon of *Escherichia coli*. J. Mol. Biol. **74**:163-177.
3. Duesberg, P. H., and P. K. Vogt. 1973. Gel electrophoresis of avian leukosis and sarcoma viral RNA in formamide: comparison with other viral and cellular species. J. Virol. **12**:594-599.
4. Ehrenfeld, E., and D. Summers. 1972. Adenylate-rich sequences in vesicular stomatitis virus messenger ribonucleic acid. J. Virol. **10**:683-688.
5. Huang, A., D. Baltimore, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. Virology **42**:946-957.
6. Huang, A., J. Greenwalt, and R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. I. Preparation, morphology and some biological properties. Virology **30**:161-172.
7. Huang, A., and R. Wagner. 1966. Comparative sedimentation coefficients of RNA extracted from plaque-forming and defective particles of vesicular stomatitis virus. J. Mol. Biol. **22**:381-384.
8. Morrison, T., M. Stampfer, D. Baltimore, and H. F. Lodish. 1974. Translation of vesicular stomatitis messenger RNA by extracts from mammalian and plant cells. J. Virol. **13**:62-72.
9. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. **17**:117-130.
10. Petermann, M. L., and A. Pavlovec. 1966. The subunits and structural ribonucleic acids of Jensen sarcoma ribosomes. Biochim. Biophys. Acta **114**:264-276.
11. Repik, P., and D. H. L. Bishop. 1973. Determination of molecular weights of animal RNA viral genomes by nuclease digestion. I. Vesicular stomatitis virus and its defective T particle. J. Virol. **12**:969-983.
12. Schincariol, A., and A. Howatson. 1972. Replication of vesicular stomatitis virus. II. Separation and characterization of virus specific RNA species. Virology **49**:766-783.
13. Soria, M., and A. Huang. 1973. Association of poly(A) with mRNA of VSV. J. Mol. Biol. **77**:449-455.
14. Stampfer, M., D. Baltimore, and A. Huang. 1971. Absence of interference during high multiplicity infection by clonally purified vesicular stomatitis virus. J. Virol. **7**:407-411.
15. Stampfer, M., A. Huang, and D. Baltimore. 1969. Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. J. Virol. **4**:154-161.
16. Stanley, W. M., Jr., and R. M. Bock. 1965. Isolation and physical properties of the ribosomal ribonucleic acid of *Escherichia coli*. Biochemistry **4**:1302-1311.
17. Wagner, R., A. Levy, R. Snyder, G. Ratcliff, and D. Hyatt. 1963. Biological properties of two plaque variants of vesicular stomatitis virus. J. Immunol. **91**:112-122.