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RNA Virus Genomes Hybridize to Cellular rRNAs and to Each Other

MARCELLA A. McCLURE[1]†* AND JACQUES PERRAULT[2]‡

Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110[1] and Molecular Biology Institute and Biology Department, San Diego State University, San Diego, California 92182[2]

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In this communication we show that the RNA genomes of vesicular stomatitis, Sindbis, and reoviruses can specifically hybridize under stringent conditions to the large rRNAs present in HeLa cell cytoplasmic extracts. In addition, we show that some virus genome RNAs can also hybridize to each other. On the basis of our previous detailed studies identifying specific regions of hybridization between the poliovirus genome and 28S rRNA, we suggest that a similar phenomenon of "patchy complementary" may be responsible for the interactions described here (M. A. McClure and J. Perrault, Nucleic Acids Res. 13:6797–6816, 1985). The possible biological implications of these cross-reacting hybridizations and practical considerations in the use of viral probes for diagnosis are discussed.

We have recently demonstrated that RNAs from various picornaviruses (poliovirus, mengovirus, and coxsackievirus) can hybridize specifically to the large rRNAs of higher eucaryotes but not to those of lower eucaryotes (yeasts) or procaryotes and that this signal cannot be accounted for merely by hybridization to regions of high G/C content (8). In this same study, we showed that three or more regions are involved in the hybridization between the poliovirus genome and 28S rRNA. A logical extension of this novel finding was to assay other RNA viruses for their ability to hybridize to rRNAs.

To address this question, we chose three unrelated viruses representative of different replicative strategies: Sindbis virus, a togavirus with a large single-stranded genome of plus-strand polarity (11.7 kilobases); reovirus, the prototype of a class of viruses with segmented, double-stranded genomes (in this case, 10 individual genome segments falling into three size classes designated L, M, and S; 23 kilobase pairs total); and vesicular stomatitis virus (VSV), a large single-stranded genome of minus-strand polarity (11.2 kilobases) (3). We document here that the genomes of these three viruses are capable of hybridizing under stringent conditions to large rRNAs from HeLa cells. In addition, we show that reovirus and Sindbis virus genomes can also hybridize to poliovirus RNA. In light of these results, we assess the possible biological significance of these RNA:RNA hybridization interactions, and we suggest caution in the use of hybridization probes corresponding to RNA virus genome sequences for detecting the presence of viral agents in cells and tissues.

MATERIALS AND METHODS

Poliovirus was grown and purified as previously described (7). VSV genomic RNA and in vitro transcription products were obtained as outlined earlier (9). Poly(A)$^+$ selection of these transcription products was carried out using oligo(dT)-cellulose (Bethesda Research Laboratories, Gaithersburg, Md.) at room temperature with a high-salt binding buffer (1 M KCl; 0.01 M Tris chloride, pH 7.5; 0.5% Sarkosyl; 1 mM EDTA) and low-salt elution buffer (0.01 M Tris chloride, pH 7.5; 0.5% Sarkosyl; 1 mM EDTA). The sample was denatured by boiling 1 min in TE (0.01 M Tris chloride, pH 7.5; 1 mM EDTA), quickly cooled in an ice slurry, and made 3 M KCl before being bound to the column. Sindbis virus RNAs (49S and 26S) [extracted from crude virus pellets from infected cell lysates and selected on oligo(dT)-cellulose] and reovirus genomic RNAs were gifts from S. Schlesinger and R. Thach, respectively (Washington University School of Medicine). *Sma*I fragments of adenovirus type 2 were a gift from R. Roeder (Rockefeller University, New York, N.Y.). Cytoplasmic extracts of HeLa cells containing ~80 to 90% rRNAs were obtained as described previously (8).

Glyoxal denaturation of RNA samples, agarose gel separation, the subsequent electrophoretic transfer of nucleic acids to nitrocellulose paper, and the procedure for labeling the fragmented RNA probes (modal distribution, 75 nucleotides) were also described previously (8). The specific activity of the viral RNA probes ranged from ca. 14×10^6 to 23×10^6 cpm/μg, and $\sim 5 \times 10^6$ cpm was used on each blot. Hybridization was carried out for 48 h at 42°C in 4× SSC (0.6 M NaCl, 0.06 M sodium citrate)–0.04 M sodium phosphate (pH 6.5)–soluble yeast RNA (Sigma type 6) at 150 μg/ml–0.16% sodium dodecyl sulfate–48% formamide–10% dextran sulfate (Pharmacia), and the final blot washes were carried out in 0.1× SSC–0.1% sodium dodecyl sulfate and 60°C as described in detail previously (8).

RESULTS

Hybridization between viral RNA genomes and rRNAs. Using the Northern blot protocol and hybridization conditions described in our previous studies with poliovirus (see above), we tested whether various viral RNAs could hybridize to the rRNAs contained in HeLa cell extracts. To facilitate the descriptions of hybridization between the molecules assayed in this study, the following notation will be used: labeled probe molecule in solution/unlabeled molecule bound to nitrocellulose paper. For example, reo/28S refers to labeled reovirus RNA probing unlabeled 28S rRNA.

Figure 1 shows the results obtained with four identical samples of RNA from HeLa cell cytoplasm when probed by

* Corresponding author.

† Present address: Department of Chemistry, D-006, University of California, San Diego, La Jolla, CA 92093.

‡ Present address: Biology Department, San Diego State University, San Diego, CA 92182.

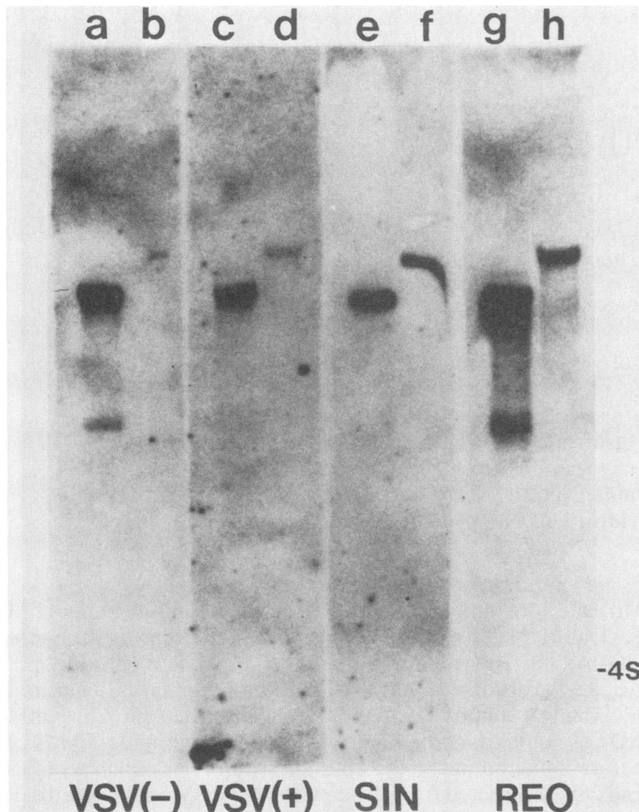

FIG. 1. Northern blot analysis of cytoplasmic RNAs from HeLa cell extracts and purified poliovirus genomic RNA probed by labeled virus RNAs. Four identical cytoplasmic RNA samples (lanes a, c, e, and g) and four identical poliovirus RNA samples (lanes b, d, f, and h), each containing ~200 ng, were probed with the labeled viral RNA probes indicated, as described in the text. The position of cellular 4S RNA is indicated.

minus-strand VSV genomic RNA (lane a), poly(A)$^+$ selected VSV mRNAs (lane c), Sindbis virus RNA (lane e), and reovirus genomic RNAs (lane g). The specific activity and amounts of each RNA probe used on each blot were approximately equal (see Materials and Methods). Clearly, these RNA virus genomes elicited a hybridization signal specific for large rRNAs when used to probe these uninfected cytoplasmic extracts. However, the signal varied among the viruses. The signal was strongest for reo/28S (Fig. 1, lane g), VSV(−)/28S (lane a), and Sindbis/28S (lane e), followed by VSV(+)/28S (lane c), reo/18S (lane g), and VSV(−)/18S (lane a). No signals were detected for VSV(+)/18S (lane c), or Sindbis/18S (lane e). Note that the VSV mRNA probe [VSV(+)] consisted of in vitro synthesized transcripts (see Materials and Methods) and therefore did not represent equimolar amounts of mRNAs, but reflected the attenuated transcription of the genes of this virus in the order N > NS > M > G > L. The hybridization signal between these molecules and 28S rRNAs most likely reflects the properties of one or more of the abundant mRNAs (N, NS, or M). No hybridization to small cellular RNAs was detected for all the virus genomes studied here (Fig. 1). In our previous studies with poliovirus, the small cellular RNAs remained negative whether or not yeast soluble RNA was included as carrier for the labeled probe.

To verify the authenticity of these hybridization signals, we carried out the converse experiments using labeled rRNA (i.e., uninfected, labeled HeLa cell cytoplasmic RNA extracts, which are estimated to contain 80 to 90% rRNAs) as the probe and agarose gel-fractionated viral RNAs. Figure 2A, lanes a, b, and c, respectively, contained equal amounts (~250 ng) of VSV, Sindbis virus, and reovirus RNAs. Clearly no signal was detected for any rRNAs which might have been contaminating these viral RNA preparations. Such contamination would have been readily detected since fragmented rRNAs efficiently probe intact 28S and 28S species under these conditions because of their high degree of secondary structure (M. A. McClure, Ph.D. thesis, Washington University, St. Louis, Mo., 1984).

Interestingly, the strongest hybridization signal was detected for the largest class of reovirus segments, rRNA/reo(L), while little or no signal could be detected for the middle-size [rRNA/reo(M)] and small-size [rRNA/reo(S)] classes (Fig. 2, lane c). Note that the individual reovirus segments within each size class were not resolved in this agarose gel analysis, and we therefore cannot deduce whether one, two, or all three L segments react with rRNAs. Figure 2B shows a control experiment where all three size

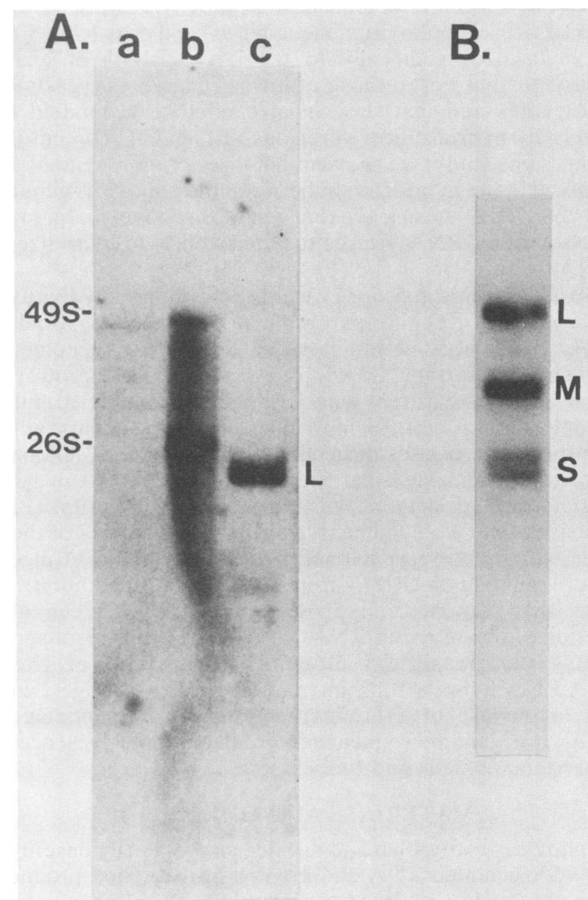

FIG. 2. Northern blot analysis of VSV, Sindbis virus, and reovirus RNAs probed by rRNAs from HeLa cells. (A) Lanes a, b, and c contained ~200 ng each of VSV, Sindbis virus, and reovirus RNAs probed by labeled RNA from a HeLa cell cytoplasmic extract as described in the legend of Fig. 1. The positions of 49S and 26S Sindbis virus RNAs and the reovirus large segment (L) are indicated. (B) A similar Northern blot analysis of unlabeled reovirus RNA (~200 ng) probed with itself. The positions of the large (L), medium (M), and small (S) segments are indicated.

classes of unlabeled reovirus segments could be probed by labeled reovirus RNA, thereby demonstrating that these molecules were efficiently transferred and retained throughout the hybridization process.

A relatively strong signal was also detected for both 26S subgenomic and 49S genomic size Sindbis virus RNA (Fig. 2A, lane b) in proportion to their relative amounts in the preparation (see Materials and Methods) when probed with labeled cytoplasmic RNA. This finding suggests that the position of this complementary interaction is either contained within the 3'-end one-third of the genome represented by the 26S subgenomic RNA, or distributed over at least two distinct regions, corresponding to the 5' two-thirds and the 3' one-third of the 49S Sindbis RNA (10).

Little signal could be detected for VSV genomic RNA when probed with labeled HeLa RNA (Fig. 2A, lane a). It is noteworthy that a strong hybridization signal was obtained in the VSV(−)/28S rRNA experiment (Fig. 1, lane a) but not in this rRNAs/VSV(−) converse experiment despite repeated attempts. A possible explanation for this seemingly paradoxical finding is discussed below.

Hybridization between viral RNA genomes. Since all of the RNA virus genomes examined here hybridized to at least one species of rRNA, we explored whether viral RNAs could also hybridize to each other. Lanes b, d, f, and h of Fig. 1 show the results obtained with the various labeled viral RNA probes tested with unlabeled poliovirus RNA analyzed on the same gel as the rRNAs. The reo/polio and Sindbis/polio reactions were clearly positive, while little or no signal was observed for VSV(−)/polio and VSV(+)/polio.

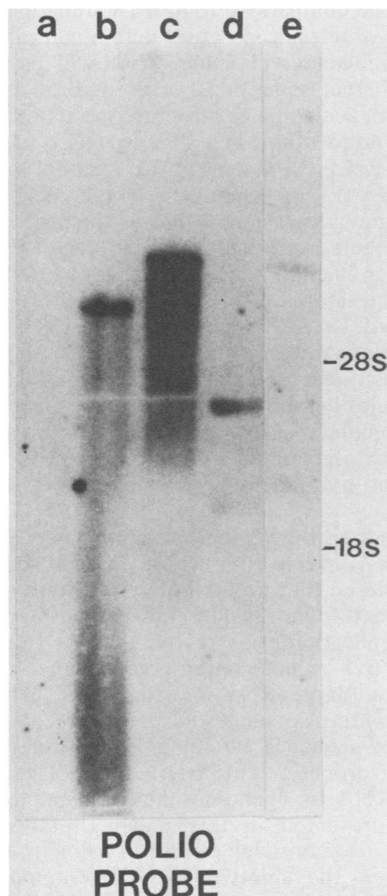

FIG. 3. Northern blot analysis of virus genomes probed by poliovirus RNA. Lane a, Small digested DNA fragments of adenovirus type 2 (~800 ng); lane b, poliovirus RNA (~200 ng); lane c, Sindbis virus RNAs (~200 ng); lane d, reovirus RNAs (~200 ng); lane e, VSV genomic RNA (~200 ng). Analysis was carried out as described in the legend of Fig. 1. The positions of HeLa cell 28S and 18S rRNAs, run on a parallel lane, are indicated.

TABLE 1. Detection of hybridization between labeled virus RNAs and unlabeled HeLa cellular RNAs

Labeled virus RNA probe	Hybridization[a] with cellular RNAs:		
	28S	18S	4–5.8S
Polio	+	+	−
Sindbis	+	−	−
VSV(−)	+	(±)	−
VSC(+)	+	−	−
Reo	+	+	−

[a] Positive (+) hybridization reactions are defined as signals which are at least two orders of magnitude stronger than negative (−) reactions (see text), and (±) refers to a signal intermediate between + and −.

The results of the converse experiment, using labeled poliovirus RNA as a probe, are presented in Fig. 3. A signal of comparable strength was detected for polio/polio (self; Fig. 3, lane b), polio/Sindbis 49S (lane c), and polio/reo (L) (lane d). No significant signal was observed for polio/VSV(−) (lane e), polio/Sindbis 26S (lane c), or polio/reo(M) and polio/reo(S) (lane d). In addition, lane a contained a *Sma*I digest of adenovirus type 2 DNA which was also negative for hybridization to the poliovirus probe, although all of the restriction fragments were efficiently transferred and retained on nitrocellulose paper throughout the procedure under these conditions (data not shown). Note that we have previously shown that the poliovirus RNA probe does react with genomic DNA representing the 28S rRNA gene under these conditions of hybridization (8), and thus the absence of a signal with adenovirus DNA is not due to the lower T_m of DNA-DNA versus DNA-RNA hybrids (2).

Relative strength of hybridization signals detected with rRNAs and viral RNAs. Previous quantitation by densitometric scanning of the poliovirus RNA hybridization signal to various RNAs revealed that equivalent amounts of VSV, yeast, and procaryotic rRNAs react at least 50- to 100-fold less strongly with labeled poliovirus rRNA than with 28S rRNA of higher eucaryotes. Similar quantitation of the signals reported in this study shows that the polio/polio, polio/Sindbis 49S, and polio/reo(L) signals (Fig. 3) were slightly weaker than that of the polio/28S rRNA (not shown). In the converse experiment, using labeled reovirus and Sindbis virus RNAs to probe poliovirus RNA, the signals were also slightly less than those found when these viral RNAs were used to probe 28S rRNA (Fig. 1, lane f and h versus lanes e and g).

Although small variations in hybridization signals were detected when the same nonogram amounts of different virus RNA samples were compared, it is nonetheless clear that almost all of the signals (except one) could be characterized as either strong or very weak, i.e., at least two orders of magnitude lower on an equimolar basis, or undetectable. Note that relative molar amounts of the RNA virus genomes used in these experiments did not differ from each other by more than a factor of 3, which is small compared to the 50- to 100-fold difference between positive and negative signals (the largest difference in molecular weight is between

TABLE 2. Detection of hybridization between labeled poliovirus genome RNA or labeled uninfected HeLa cytoplasmic RNA (rRNA) and unlabeled virus RNAs

Labeled probe	Hybridization[a] with virus RNAs:						
	Polio	VSV	Sindbis		Reo		
			49S	26S	L	M	S
Poliovirus	+	−	+	−	+	−	−
rRNA	+	−	+	+	+	−	−

[a] Positive (+) and negative (−) reactions are defined as in Table 1, footnote *a*.

reovirus and poliovirus). Furthermore, we have verified that the rRNA/polio signal is roughly linear over the range of at least 5 to 500 ng of material blotted onto nitrocellulose (not shown). For comparison purposes we have therefore defined the strong signals as positive (+) while both very weak signals and undetectable ones are defined as negative (−). The results are summarized in Tables 1 and 2.

DISCUSSION

We demonstrate in this paper that the hybridization phenomenon we detailed previously between poliovirus RNA and large rRNAs (8) is not unique to picornaviruses. Genomic RNAs of VSV, Sindbis virus, and reovirus hybridize under stringent conditions to the large rRNAs extracted from the cytoplasm of HeLa cells. Although all of these viral RNAs appear capable of forming stable hybrids with 28S rRNA, only reovirus and possibly VSV, hybridize, albeit less strongly, to 18S rRNA. Interestingly, only the large class of segments of reovirus hybridizes significantly to rRNAs, whereas both the complete 49S genomic and 26S subgenomic RNAs of Sindbis virus contain regions complementary to 28S rRNA (for summary see Tables 1 and 2).

The second set of experiments described in this paper explored whether RNA virus genomes can also hybridize to each other. Although only some of the various pairwise combinations were tested, the results show that sequences found among the largest RNA segments of reovirus and 49S Sinbdis virus genome RNA, but not 26S, can elicit a strong hybridization signal when probed by poliovirus RNA (see Table 2).

In the following discussion we will address several points that we consider to be of importance in evaluating these studies. First, it should be emphasized that while a number of different RNA molecules possess the capacity to base-pair with one another under stringent hybridization conditions, not all RNA molecules examined here are capable of this interaction. No signal could be detected from the viral RNA/cellular 4 to 5.8S RNAs, Sindbis/18S rRNA, rRNAs/reo (middle or small segments), polio/VSV(−), or polio/Sindbis 26S combinations. In our previous studies detailing poliovirus RNA hybridization to rRNAs we showed that this virus genome could not hybridize significantly to rRNAs of yeasts or procaryotes (this has not yet been tested for the other viral RNAs considered here). Furthermore, under conditions where we could detect hybridization between poliovirus RNA and specific restriction fragments from 28S rDNA genes, we found no signal with pBR322 or λ phage DNA or, as shown here, with adenovirus type 2 DNA (Fig. 3, lane a). Even lowering the stringency of the hybridization conditions did not reveal any with pBR322 or adenovirus type 2 DNA (McClure, thesis). Therefore, as far as the polio/28S rRNA signal is concerned, specificity is not due to the fact that RNA:RNA hybrids are more stable than RNA:DNA. We have no reason to suspect at the present that the signals reported in this communication are qualitatively different, although more detailed characterization is in order.

The hybridization we detect is also not likely to be due simply to a high probability of such interactions between large-size RNA molecules, since not all species examined possess this ability, and the relative strengths of the signals do not correlate with size. Whether or not regions of high G/C content are solely responsible for the particular hybridization reactions we detect here will also require further study. In the case of poliovirus and 28S rRNA, several lines of evidence have led us to conclude that this is clearly not the case. Moreover, one particular region of complementarity between these molecules which we characterized in detail could only be accounted for by a hybrid structure of "patchy" complementarity where 81 out of 104 contiguous bases of rRNA were involved (8). Since the studies here were carried out using the same hybridization conditions and resulted in signals of comparable magnitude, similar types of patchy hybrid structures may be responsible for all these virus-to-virus and virus-to-rRNA signals. The fact that poliovirus genomic RNA also self-hybridizes under these conditions suggests that the stability of the intermolecular hybrids may be comparable to at least some major domains of secondary or tertiary structure within naturally occurring large RNA molecules. Further work will be required to determine whether some or all of the hybridization regions involved share sequence or structure motifs or both.

It is worth noting that when VSV was used to probe HeLa cytoplasmic RNAs, a strong positive signal was obtained with 28S rRNA (Fig. 1, lane a). In the converse experiment, however, using cytoplasmic RNAs as the probe, little or no signal was detected with VSV genomic RNA (Fig. 2, lane a). A similar phenomenon occurred when we tried to probe a restriction digest of the infectious cDNA of polio with a nick-translated DNA fragment from the 28S rRNA gene which had been shown to hybridize in the converse experiment. As suggested previously (8), it is possible that large RNA molecules bound to nitrocellulose paper assume a long-range secondary structure involving regions of the molecule which would otherwise be available for intermolecular hybridization in the context of a smaller fragment.

With respect to the biological significance of the cross-hybridization between viral RNAs and rRNAs, we have previously offered four possibilities: (i) distant evolutionary ancestry, (ii) a role in translation or utilization of ribosomal factors in replication, (iii) recombination and adaptation events, and (iv) complementary secondary structure domains between large RNA molecules (8). The first two possibilities were also suggested before in connection with the finding of sequence homologies between RNA phage genomes and procaryotic 16S rRNA (4). The interactions detected for RNA viral genomes that are capable of acting as messengers presented in this paper (Sindbis virus and reovirus) are also consistent with a possible role in translation. However, the ability of the VSV minus strand to hybridize to 28S rRNA suggests that some other phenomenon may be involved.

As far as cross-hybridization between RNA virus genomes is concerned, recent computer-assisted comparisons of the proteins of several RNA viruses show that viruses from the same class are closely related, as expected, while some distant relationships may exist between classes (1, 5, 6). Our

results suggest that relationships between classes of RNA viruses may also be seen at the nucleotide level.

In light of our studies, we again advise caution in the use of probes representing RNA genomes for the detection of viral agents in infected tissue and cells, whether it be for diagnostic or other purposes. In most cases, such probes should correspond only to regions of the genomes which do not cross-react to rRNAs. Extensive testing will also be necessary to avoid confusion in the identification of particular infectious agents since some RNA viral genomes hybridize to each other.

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