Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3522–3526, May 1988
Genetics

Analysis of genetic variability and mapping of point mutations in influenza virus by the RNase A mismatch cleavage method

(RNA virus/genetic divergence)

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Communicated by Severo Ochoa, January 19, 1988

ABSTRACT **We have applied the RNase A mismatch cleavage method to analyze genetic variability in RNA viruses by using influenza virus as a model system. Uniformly labeled RNA probes synthesized from a cloned hemagglutinin gene of a given viral strain were hybridized to RNA isolated from other strains of characterized or uncharacterized genetic composition. The RNA·RNA heteroduplexes containing a variable number of base mismatches were digested with RNase A, and the resistant products were analyzed by denaturing polyacrylamide gel electrophoresis. We show that many of these single base mismatches are cleaved by RNase A, generating unique and characteristic patterns of resistant RNA fragments specific for each of the different viral strains. Comparative analysis of the cleavage patterns allows a qualitative estimation of the genetic relatedness and evolution of field strains. We also show that cleavage by RNase A at single base mismatches can readily detect and localize point mutations present in monoclonal antibody-resistant variants. This method should have wide applications in the study of RNA viruses, not only for epidemiological analysis but also in some diagnostic problems, such as characterization of phenotypic mutants.**

RNA viruses show a high spontaneous mutation rate, which allows the rapid evolution of viral populations (1) and the existence of extensive genetic heterogeneity with important biological consequences (2). In many instances, one or a few point mutations have been shown to lead to essential phenotypic changes. Some relevant examples include the outcome of epidemic variants of influenza or foot and mouth disease viruses (antigenic drift) (3), changes in tropism (4) or in pathogenicity (5) of viruses, or isolation of variants resistant to neutralizing monoclonal antibodies (6).

A variety of techniques have been used for the detection and study of genetic variation among RNA viruses. Some of them are based on migration differences that under certain electrophoretic conditions can be observed with single- (7) or double-stranded (8) RNAs differing in one or a few nucleotides. Similarly, conditions of partial denaturation can be used to distinguish RNA·RNA or DNA·RNA heteroduplexes from the corresponding homoduplexes (9, 10). However, these techniques do not discriminate between single or multiple nucleotide changes and also require experimental conditions that have to be empirically determined in each case. RNase T1 oligonucleotide fingerprinting has been useful in many studies of viral heterogeneity and evolution (11). This technique yields quantitative data but becomes cumbersome in the analysis of multiple samples. Nucleotide sequencing is widely used for genetic analysis of RNA viruses and has opened insights into variation studies (12). However, sequencing of large viral genomes or of multiple viral isolates represents a formidable experimental task. Therefore, a simple technique that could detect point mutations in viral RNA genomes would be very useful for this type of study.

We developed a method to detect single base substitutions in transcribed genes that is based on the ability of RNase A to cleave single base mismatches in RNA·RNA heteroduplexes (13). The method has been successfully applied to detect mutated cellular *KRAS* oncogenes in human tumors (13, 14) and single mutations in the human hypoxanthine-guanine phosphoribosyltransferase gene (15) and in the mouse ornithine carbamoyltransferase gene (16). In addition, single base substitutions in the β-globin gene have been detected in β-thalassemias by RNase A cleavage of DNA·RNA heteroduplexes containing single base mismatches (17).

Using influenza virus as a model system, we show that the RNase A mismatch cleavage method provides an alternative and simple approach for analysis of genetic variability in RNA viruses. We also show that cleavage by RNase A at single base mismatches in viral RNA hybrids can readily detect and localize single point mutations in monoclonal antibody-resistant variants.

MATERIALS AND METHODS

Viral Strains and RNAs. The viral strains used throughout this work (with their abbreviated names in parenthesis) are as follows: A/Hong Kong/8/68 (HK68), A/England/42/72 (EN72), A/Victoria/3/75 (VA75), A/Texas/1/77 (TX77), A/Philipines/2/82 (FP82), A/Brazil/11/78 (H1), and A/England/12/64 (H2). Strains A/Madrid/9688/80 (MA80) and A/Madrid/716/81 (MA81) were isolated 2 months apart during the same influenza outbreak. Both isolates have been characterized by T1 fingerprinting (18). Mutants RM1 (M1), RM2 (M2), and RM4 (M4) are viruses resistant to neutralization by M58/p7/C anti-hemagglutinin monoclonal antibody (19). These monoclonal antibody-resistant variants were obtained from the VA75 strain after selection by two consecutive plaque isolation steps in the presence of antibody.

Viruses were grown in either 10-day-old embryonated chicken eggs or in cultures of Madin–Darby canine kidney (MDCK) cells. They were partially purified by precipitation with 10% PEG-6000 followed by centrifugation through a 30% sucrose gradient (19). Viral RNAs were obtained from purified viruses as described (20). Total cellular RNA from MDCK-infected cells was isolated from subconfluent cultures infected at high multiplicity. Twenty-four hours after infection, cell monolayers were harvested and RNA was

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prepared by the guanidine hydrochloride method as described (13).

Plasmids. Plasmid pSVa970 contains a full-length cDNA sequence of the influenza virus hemagglutinin gene from the VA75 strain cloned into the pBSV9 vector (21). pSVa970 was digested with *Stu* I (which cuts in the early promoter of simian virus 40, 20 nucleotides from the 5′ end of the viral HA) and *Eco*RI (at position 1272 in the hemagglutinin gene). The *Stu* I/*Eco*RI fragment was subcloned into the *Sma* I and *Eco*RI sites of pGEM3 (Promega Biotec, Madison, WI), generating pIVAV1. From this recombinant, deletion plasmids were obtained by digestion with *Hin*dIII (pIVAV3), *Xba* I (pIVAV4), and *Pst* I (pIVAV5), and ligation with T4 DNA ligase. The 523-nucleotide *Taq* I fragment from the BK79 HA sequence (positions 676–1199) was isolated from plasmid pBK4 (22) and was cloned into the *Acc* I site of pGEM3, generating pIVAB1.

Hybridizations and RNase Digestions. Plasmid DNA (1 μg), digested with the appropriate enzyme, was used for the preparation of uniformly labeled RNA probes with either SP6 or T7 RNA polymerases as described (13) using ^{32}P-labeled CTP (Amersham). For short probes (<400 nucleotides), 24 μM unlabeled CTP was used in the reaction mixture and incubation was for 1 hr, whereas for longer probes the amount of unlabeled CTP was raised to 500 μM and incubation was for 2 hr. Uniformly labeled RNA probes were purified by preparative electrophoresis in 5% polyacrylamide gels and hybridized (7×10^4 cpm) to 1–5 μg of either viral RNA or total cellular RNA from infected cells, for 3.5 hr at 57°C, as described (13). Hybridization with viral RNA from H1N1 and H3N2 reference strains was done at 40°C. The hybrids were digested with RNase A (60 μg/ml) (P-L Biochemicals) and RNase T1 (2 μg/ml) (Sigma) for 1 hr at 30°C. RNase T1 was added to achieve a more extensive digestion of small fragments of the nonhybridized RNA probes. The products were analyzed by electrophoresis in 8% denaturing polyacrylamide gels and autoradiography as described (13).

RESULTS

RNase A Mismatch Cleavage Analysis of Influenza Virus Genetic Variants. We selected a fragment of the influenza virus hemagglutinin gene spanning positions 1–1272. This region encodes the major antigenic determinants of the hemagglutinin molecule and exhibits considerable sequence heterogeneity between different viral strains (Fig. 1 *Upper*). RNA probes complementary to this region of the VA75 strain genome (Fig. 1 *Upper*) were hybridized to viral RNA isolated from the BK79 or from the MA80 strains. These viral strains are genetically similar as determined by T1 fingerprinting (18). The predicted mismatches present in heteroduplexes between the VA75 complementary RNA probe and the BK79 viral RNA was inferred from the known nucleotide sequence of these viruses (23). As shown in Fig. 1, most of these mismatches accumulate in the region between the *Hin*dIII and *Pst* I sites encoding the major antigenic area of the influenza virus hemagglutinin. RNAs prepared from three different VA75 subclones isolated by their resistance to neutralization by a monoclonal antibody raised against the viral hemagglutinin were also used (M1, M2, and M4). Only a few nucleotide differences were expected between these last viral RNAs and the RNA from the parental VA75 virus. The RNA hybrids were digested with RNase A and the resistant RNA fragments were analyzed by denaturing gel electrophoresis. Due to the presence of multiple mismatches in VA75–BK79 hybrids (Fig. 1 *Upper*), a complex band

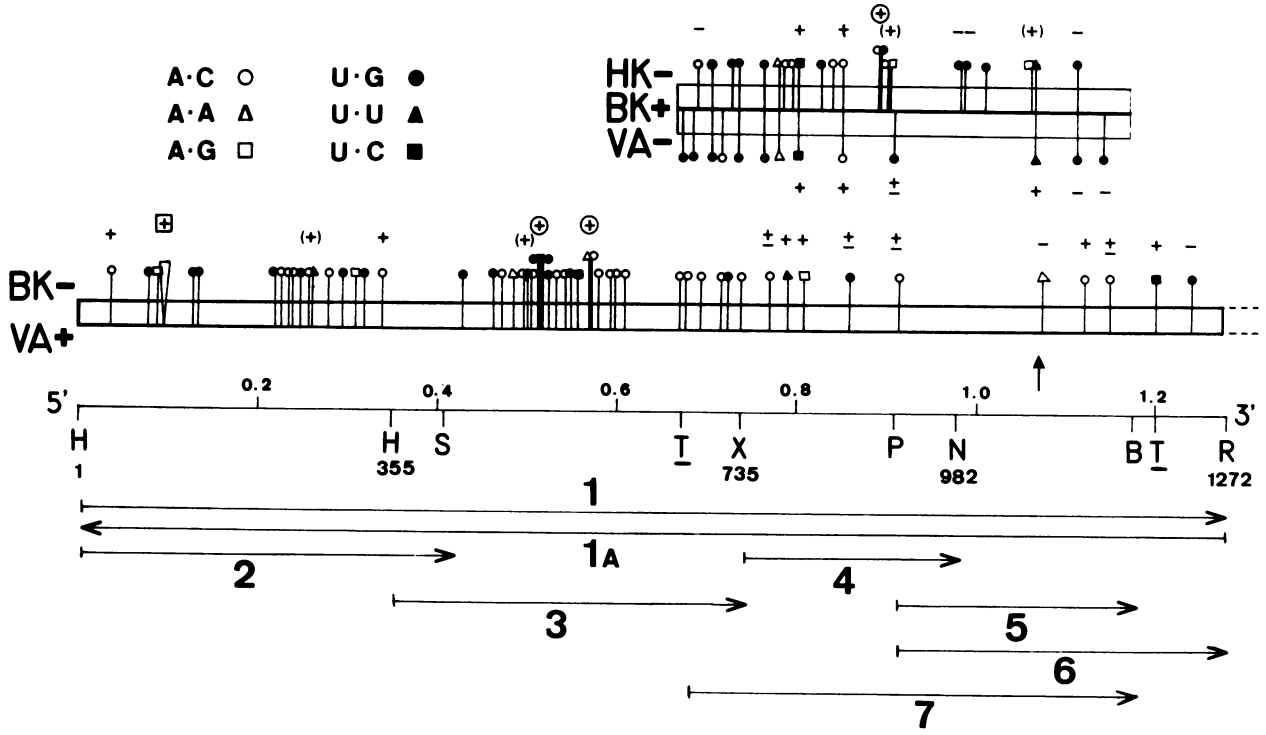

FIG. 1. Schematic representation of mismatches present in RNA heteroduplexes between the hemagglutinin genes from different influenza viruses and of the RNA probes used. (*Upper*) The predicted mismatches present in RNA heteroduplexes between the BK79 (+) probe (from positions 676–1199) and virion RNA from the HK68 and VA75 influenza virus strains and from the VA75 (+) probe (from positions 1–1272) and virion RNA from the BK79 strain. Above some of the mismatches the extent of cleavage is indicated. +, Significant cleavage (>20%); ±, partial cleavage (5–20%); −, cleavage not detected; □, deletion of 3 nucleotides; ⊕, double or triple mismatch; (+), alternate mismatches. Arrow indicates site of mutation found in monoclonal antibody resistant-variant M4. pIVAV1 was used to synthesize probes 1 and 2 with the SP6 RNA polymerase and RNA probe 1A with the T7 RNA polymerase. Probes 3 and 4 were generated by using pIVAV3 and pIVAV4, respectively. Probes 5 and 6 were synthesized by using pIVAV5 linearized with *Bgl* II or *Eco*RI, respectively. Probe 7 was synthesized by using pIVAB1. H, *Hin*dIII; S, *Spe* I; T, *Taq* I; X, *Xba* I; P, *Pst* I; N, *Nde* I; B, *Bgl* II; R, *Eco*RI.

pattern was expected with the full-length RNA probe. Therefore, we also used shorter RNA probes to generate hybrids with fewer mismatches (Fig. 1 *Lower*).

The results of these experiments are shown in Fig. 2. As expected, digestion with RNase A of the VA75-BK79 hybrids (lanes BK) resulted in a complex pattern of RNA fragments generated by RNase A cleavage at the mismatches present in the entire region (panel 1) or in the internal fragments (panels 2–6). The digestion pattern of the VA75–MA80 hybrids (lanes MA) was similar to that of the VA75–BK79 hybrids, although some differences were also apparent. In contrast, the VA75 homoduplexes were essentially resistant to digestion (lanes VA). Hybridizing smears were present below the protected bands, especially in the longer probes (panels 1–3), which were due to unspecific RNA degradation. The sizes of the RNase A-resistant fragments were larger than the sizes expected from the mismatches present in the hybrids (Fig. 1 *Upper*), indicating that not all mismatches were completely cleaved by the enzyme. The mismatch cleavage pattern was different depending on the probe used. Thus, probes 2 and 3 generated a more complex pattern than probes 4 and 6. This result was expected because the former probes covered the region of the hemagglutinin molecule where mutations accumulate more frequently (Fig. 1 *Upper*).

When virion RNA from the three monoclonal antibody-resistant mutants M1, M2, and M4, were hybridized to the full-length probe (panel 1), a single subband was observed in the hybrid corresponding to M4. This result indicates the presence of a point mutation in this viral isolate. The position

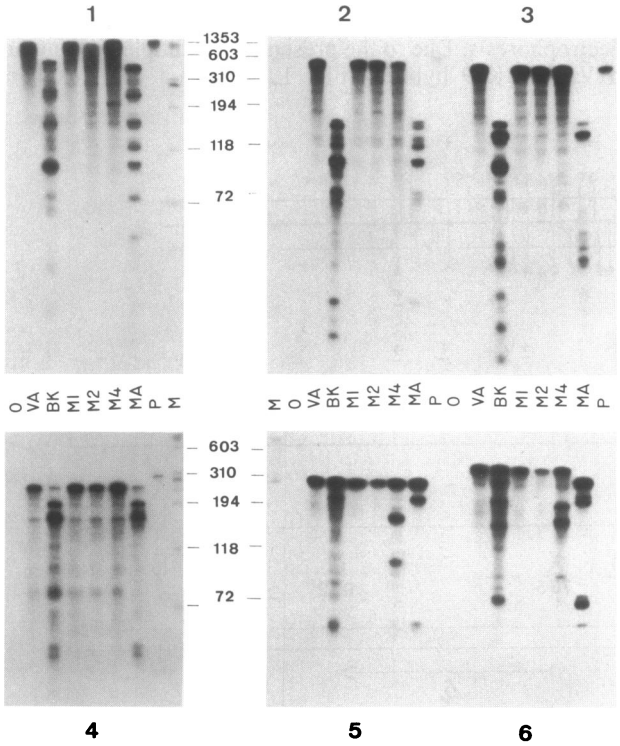

FIG. 2. RNase A mismatch cleavage analysis of RNA heteroduplexes corresponding to the influenza hemagglutinin gene from different viral strains. RNA isolated from partially purified virion particles from the viral strains indicated was hybridized to the probes indicated at the top and bottom (see Fig. 1) complementary to the noncoding RNA strand of the VA75 strain. The RNA hybrids were digested with RNase A and the resistant products were analyzed in 25-cm-long 8% denaturing polyacrylamide gels. VA, VA75; BK, BK79; MA, MA80. Lanes P and O, undigested probe and probe hybridized to carrier tRNA, respectively; lanes M, ØX174 DNA digested with *Hae* III used as molecular weight markers. Numbers in the center indicate the position and size (in nucleotides) of these fragments.

of the mutation in M4 was mapped by using two overlapping probes (Fig. 1, probes 5 and 6). These probes detected subbands of approximately 100 and 165, and 165 and 200 nucleotides, respectively (panels 5 and 6). These results localize the mutation of M4 near position 1080 in the hemagglutinin sequence. The sequence flanking this region was determined by using an oligodeoxynucleotide primer (nucleotides 1000–1018). A cytosine to guanine transversion was found at position 1076, which would lead to a phenylalanine to leucine substitution in the hemagglutinin protein sequence (data not shown). No mutations were detected for M1 and M2 with any of the probes used (Fig. 2).

These results establish the applicability of the RNase A mismatch method for the comparative analysis of the genetic relatedness of different viral strains and for the detection and localization of mutations in uncharacterized viral variants.

RNase A Mismatch Cleavage Analysis by Using Total Cellular RNA from Virus-Infected Cells. To test the possibility of using other sources of RNA instead of purified virion RNA, RNA extracted from virus-infected cells was hybridized to full-length RNA probes complementary to the coding (+) or noncoding (−) viral RNA strands (probes 1 and 1A, Fig. 1). Thus, RNA heteroduplexes could be generated between each probe and the complementary RNA corresponding to either viral RNA or mRNA (and complementary RNA) both present in the cellular RNA population. Results are shown in Fig. 3. The RNase A digestion pattern obtained when BK79 virion RNA (Fig. 3 *Left*) was hybridized to the positive polarity VA75 probe was very similar to that obtained with total cellular RNA from BK79-infected cells (Fig. 3 *Right*). This result demonstrates the reproducibility of the method and its applicability with total cellular RNA. Hybridization of total cellular RNA to RNA probe 1A, complementary to the viral coding strand, also generated a mismatch cleavage pattern that was different from the one obtained with probe 1 complementary to the noncoding viral RNA strand. This result was expected because the mismatches present in hybrids of each polarity were different. Nevertheless, some

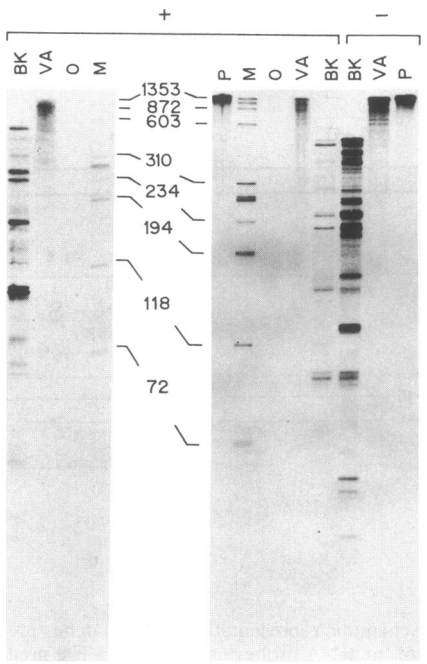

FIG. 3. RNase A mismatch cleavage analysis of influenza hemagglutinin RNA heteroduplexes using RNAs from purified virions and from infected cells. The VA75-specific probes used, 1 (+) and 1A (−) (see Fig. 1) were hybridized to RNA from purified virions (*Left*) and to total cellular RNA from virus-infected MDCK cells (*Right*). Symbols are the same as in Fig. 2.

common bands were also present, suggesting that some mismatches were cleaved in both hybrids.

Comparative RNase A Cleavage Analysis of Influenza Virus Strains. As a direct application to epidemiological studies of influenza viruses, viral RNAs from a series of reference strains of the H3N2 subtype were similarly analyzed by using the long VA75 RNA probe (Fig. 1, probe 1). Results are shown in Fig. 4. A unique and characteristic pattern of RNase A-resistant bands was obtained for each viral RNA (indicated above the lanes). Although the pattern obtained with two RNase A doses were different (Fig. 4 *Left* and *Right*), the relationships among the different viruses were internally consistent and reproducible. The complexity of the pattern correlated with the genetic relatedness of each virus. Thus, the mismatch patterns of closely related viral strains (i.e., MA80 and MA81) were very similar while the patterns of distant viral isolates were more different (i.e., HK68 compared with MA81). Strains isolated at intermediate dates exhibited intermediate differences in their mismatch patterns. Some bands were associated with genetic changes that appear or disappear at a given evolutionary point and remain unchanged thereafter. For example, HK68 and EN72 generated several common mismatch bands (arrowheads) that were not present in any of the other viral strains. On the other hand, other bands (asterisks) were present in all viral strains isolated after the VA75 strain but not in those isolated before. Based on the presence of these characteristic bands, it is possible to locate the origin of the VA75 strain between the EN72 and the TX77 strains, in agreement with their isolation date. In addition, a double band of ≈100 nucleotides (arrow) was present in all viruses and therefore represents a characteristic genetic difference of the VA75 strain. This difference corresponds to the mutational insertion of 3 nucleotides at position 101 that occurs in this virus.

These results were not dependent on the RNA probe used because similar results were obtained when a probe derived from the hemagglutinin gene from the BK79 strain (Fig. 1, probe 7) was hybridized to viral RNAs from the same strains used in the experiment of Fig. 4 and from viruses from the H1N1 and H2N2 subtypes (Fig. 5). Again, the most distant strains (i.e., HK68 and EN72) yielded the most complex pattern of mismatch-specific cleaved bands, while the closer strains generated a simpler digestion pattern. The mismatch patterns were simpler than in Fig. 4 because this probe covers a smaller region of the hemagglutinin gene containing fewer mutations (see Fig. 1). Furthermore, 9 of the mismatches present in BK79–VA75 hybrids using this probe are G·U mismatches (Fig. 1 *Upper*), which normally are not cleaved (14). Thus, the TX77, MA80, and MA81 strains exhibited a single detectable mismatch in this region. Nevertheless, the conclusions derived from results obtained with the short BK79 probe were similar to those obtained with the long VA75 probe.

No bands were observed with RNA from the H1 and H2 viruses, probably because the divergence in their nucleotide sequences prevented their hybridization to the BK79 RNA probe.

DISCUSSION

We have applied the RNase A mismatch detection method to analysis of genetic variability in RNA viruses. We have chosen influenza virus as a model system because the nucleotide sequences of various strains are well characterized. We have shown that this technique is useful for classification of viruses and for the study of evolution in viral populations during an epidemiological period. Our results on the genetic relatedness of several viruses of the H3N2 subtype (Figs. 4 and 5) are consistent with those obtained by RNase T1 fingerprinting (20) or nucleotide sequencing (23). Our method represents an alternative and complementary approach to these methods and offers the advantage of its

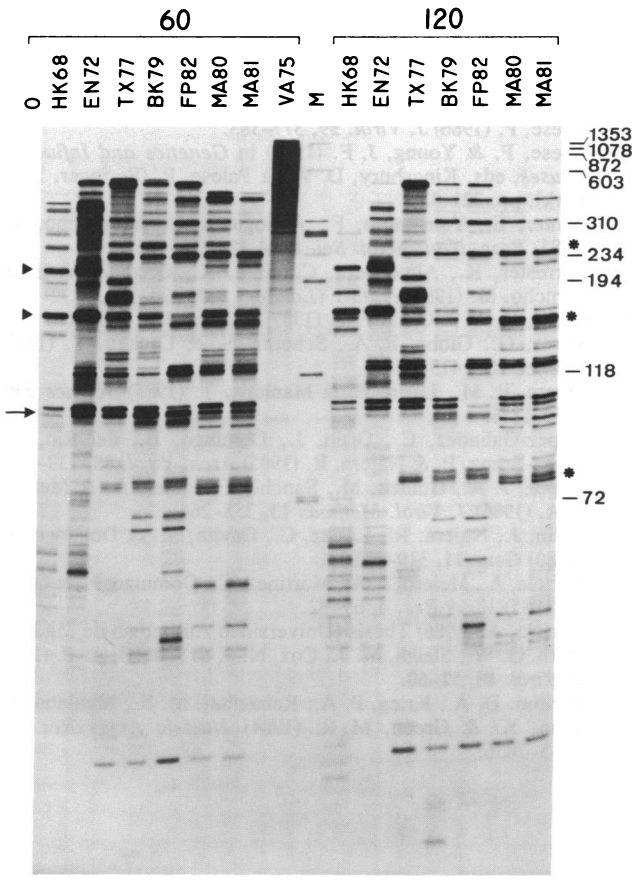

FIG. 4. Comparative RNase A mismatch cleavage analysis of influenza viruses from the H3N2 subtype. Viral RNAs isolated from the reference viruses indicated at the top were hybridized to the VA75-specific RNA probe 1 (Fig. 1). The hybrids were digested with 60 or 120 μg of RNase A per ml (indicated at the top) and the products were analyzed in a 40-cm-long 8% denaturing polyacrylamide gel. Arrow, arrowheads, and asterisks are explained in text. The rest of the symbols are the same as in Fig. 2.

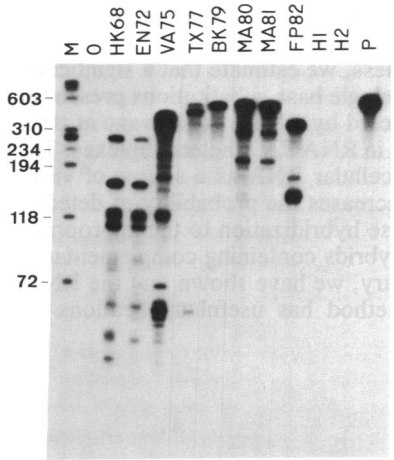

FIG. 5. RNase A mismatch cleavage analysis of influenza viruses of the H1N1, H2N2, and H3N2 subtypes. The probe (Fig. 1, probe 7) corresponds to the BK79 strain and was synthesized by using pIVAB1. The probe was hybridized to virion RNA from the same viruses of Fig. 4 and from the reference viruses H1 and H2, representative of the H1N1 and H2N2 subtypes, respectively. Symbols are the same as in Fig. 2.

simplicity. In addition, the use of total cellular RNA from virus-infected cells permits a rapid and simultaneous screening of multiple viral isolates.

In our studies, we have selected conditions of partial mismatch cleavage as can be deduced from the size of the RNase A-resistant fragments and the differences between the digestion patterns obtained with two RNase A doses. More extensive digestion can be obtained by increasing the amount of enzyme and the incubation time. However, the band patterns obtained in these conditions were complex (data not shown), and we have chosen conditions of partial cleavage because the results are easier to interpret. Nevertheless, for the same digestion conditions, the cleavage patterns were always unique and specific for each of the different viral strains, indicating the reproducibility of the method. However, partial mismatch cleavage makes difficult the quantification of the differences among viruses and the identification of the mismatches recognized. Therefore, this technique is better adapted for qualitative studies of variation in RNA viruses.

The technique is dependent on the synthesis *in vitro* of RNA probes of defined length (24). In a typical experiment, we obtain RNA probe sufficient for analysis of 50–250 samples. In addition, we have used probes that were stored at −70°C up to 2 months with the same results. We have prepared probes of up to 1350 nucleotides, and longer probes also may be synthesized. This is important because the use of long probes facilitates the screening of large genomic regions.

The RNase A method is based on the ability of this enzyme to recognize and cleave single base mismatches in RNA hybrids. Thus, we have shown that single point mutations in uncharacterized viral strains can be readily detected and localized within a few nucleotides, as with M4. However, and in agreement with previous results using RNA·RNA (13, 14) and DNA·RNA (17) heteroduplexes, our results with influenza virus RNA hybrids indicate that neither are all mismatches cleaved, nor are all equally recognized. Thus, all U·U (3), U·C (3), A·G (1), and C·C (1), only 6 of 9 A·C, and 1 of 2 A·A mismatches that we were able to identify in the RNA hybrids were recognized and cleaved to a significant extent. Furthermore, only 2 of 11 G·U mismatches were partially cleaved (Fig. 1 *Upper*). Therefore, our failure to detect mutations in monoclonal antibody-resistant variants M1 and M2 was probably due to the presence of mutations in these strains that generate mismatches that are not recognized by the enzyme.

Nevertheless, we estimate that a significant proportion of all possible single base substitutions present in RNA viruses can be detected by RNase A cleavage at the corresponding mismatches in RNA·RNA heteroduplexes. The possibility of using total cellular RNA as a source of viral RNA of both polarities increases the probability of detecting point mutations because hybridization to the appropriate RNA probes generates hybrids containing complementary mismatches.

In summary, we have shown that the RNase A mismatch cleavage method has useful applications in the study of genetic variability, epidemiology, evolution, and diagnostic characterization of phenotypic mutants of RNA viruses.

We thank C. Almoguera and C. Lama for their excellent technical assistance and K. Forrester for helpful discussions and advice. This work was supported by National Institutes of Health Grant CA33021 awarded by the National Cancer Institute to M.P. and by grants from the Comision Asesora de Investigacion Cientifica y Tecnica (Grant 608/438) and Fondo de Investigaciones Sanitarias to J.O. and J.A.M. C.L.-G. was a recipient of a NATO short-term postdoctoral fellowship while on leave from the Centro Nacional de Microbiologia, Majadahonda, Madrid.

1. Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & VandePol, S. (1982) *Science* **215**, 1577–1585.
2. Domingo, E., Martinez-Salas, E., Sobrino, F., de la Torre, J. C., Portela, A., Ortin, J., Lopez-Galindez, C., Perez-Brena, P., Villanueva, N., Najera, R., VandePol, S., Steinhauer, D., De Polo, N. & Holland, J. (1985) *Gene* **40**, 1–8.
3. Webster, R. G., Laver, W. G., Air, G. M. & Schild, G. C. (1982) *Nature (London)* **296**, 115–121.
4. Katz, J. M., Naeve, C. W. & Webster, R. G. (1987) *Virology* **156**, 386–395.
5. Almond, J. W., Stanway, G., Cann, A. J., Westrop, G. D., Evans, D. M. A., Ferguson, M., Minor, P. D., Spitz, M. & Schild, G. C. (1984) *Vaccine* **2**, 177–184.
6. Yewdell, J. W., Webster, R. G. & Gerhard, W. V. (1979) *Nature (London)* **279**, 246–248.
7. Ritchey, M. B., Palese, P. & Schulman, J. L. (1976) *J. Virol.* **20**, 307–313.
8. Ito, J. & Joklik, W. K. (1972) *Virology* **50**, 202–208.
9. Scholtissek, C. (1980) in *Structure and Variation of Influenza Virus*, eds. Laver, W. G. & Air, G. (Elsevier-North Holland, New York), pp. 191–199.
10. Smith, F. I., Parvin, J. D. & Palese, P. (1986) *Virology* **150**, 55–64.
11. Parvin, J. D., Moscona, A., Pan, W. T., Leider, J. M. & Palese, P. (1986) *J. Virol.* **59**, 377–383.
12. Palese, P. & Young, J. F. (1983) in *Genetics and Influenza Viruses*, eds. Kingsbury, D. W. & Palese, P. (Springer, New York), pp. 321–336.
13. Winter, E., Yamamoto, F., Almoguera, C. & Perucho, M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7575–7579.
14. Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. & Perucho, M. (1987) *Nature (London)* **327**, 298–303.
15. Gibbs, R. A. & Caskey, T. (1987) *Science* **236**, 303–305.
16. Veres, G., Gibbs, R. A., Scherer, S. & Caskey, T. (1987) *Science* **237**, 415–417.
17. Myers, R. M., Larin, Z. & Maniatis, T. (1985) *Science* **230**, 1242–1246.
18. Lopez-Galindez, C., Ortin, J., Domingo, E., del Rio, L., Perez-Brena, P. & Najera, R. (1985) *Arch. Virol.* **85**, 139–144.
19. Lopez, J. A., Guillen, M., Sanchez-Fauquier, A. & Melero, J. A. (1986) *J. Virol. Methods* **13**, 255–264.
20. Ortin, J., Najera, R., Lopez, C., Davila, M. & Domingo, E. (1980) *Gene* **11**, 319–331.
21. Portela, A., Melero, J. A., Martinez, C., Domingo, E. & Ortin, J. (1985) *Virus Res.* **4**, 69–82.
22. Portela, A. (1986) Thesis (Universidad Autonoma de Madrid).
23. Both, G. W., Sleigh, M. J., Cox, N. J. & Kendal, A. P. (1983) *J. Virol.* **48**, 52–60.
24. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **18**, 7035–7056.