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Analysis of Respiratory Syncytial Virus Genetic Variability with Amplified cDNAs

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Antigenic and genetic heterogeneities exist within the two major antigenic groups of respiratory syncytial (RS) virus. We developed a polymerase chain reaction (PCR)-based assay that not only differentiates the two RS virus groups but allows distinctions within groups on the basis of changes in the nucleotide sequences, as revealed by restriction fragment analysis. In this assay, viral RNA served as a template for cDNA synthesis with extension from a synthetic oligonucleotide primer complementary to bases 164 to 186 in the F protein mRNA. For PCR amplification, two group-specific 5′ primers were added. The two primers corresponded to the G protein mRNA sequence of group B (bases 10 to 30) or group A (bases 247 to 267) RS virus. Agarose gel electrophoresis readily discriminated the 1.1-kb group B and the 0.9-kb group A virus amplification products. All 47 viruses tested were assigned to the same group by both PCR and monoclonal antibody reaction pattern analysis. Restriction fragment analysis of the amplified DNAs revealed 12 restriction patterns for group A viruses and 7 restriction patterns for group B viruses, while the monoclonal antibody reaction patterns revealed seven patterns for group A viruses and 3 patterns for group B viruses. Most viruses with the same monoclonal antibody reaction patterns had different restriction patterns, and some viruses with the same restriction patterns had different monoclonal antibody reaction patterns. Thus, the results of the PCR assay concurred with the monoclonal antibody reaction pattern analysis for group classification of RS viruses, while the restriction fragment analysis identified greater diversity within groups than was seen with the monoclonal antibody analysis.

Respiratory syncytial (RS) virus is a major cause of acute respiratory tract infections in infants and young children. Two interesting and unexplained aspects of the immunobiology of RS virus are that (i) infants may be infected even in the presence of transplacentally acquired maternal antibody and (ii) reinfections are the norm and occur throughout life (18). Antigenic variation among the RS viruses may contribute to the ability of RS virus to evade the immune system in these situations. Although previously thought to be a monotypic population of viruses, it is now known that there are two major antigenic classes of RS virus; these are designated as groups, subtypes, or subgroups (3, 21). The two groups are delineated by their patterns of reactivity with monoclonal antibodies (MAbs), and the greatest differences are found on the G protein (3, 21). The most extensive amino acid sequence differences between the groups are also found on the G protein (17).

The differences between the two major antigenic groups are reflected in the immune responses of children. Infants and young children infected with RS virus develop antibody responses to the attachment glycoprotein G which are predominantly group specific, and neutralizing antibody responses to a virus of the homologous infecting group are greater than those to a virus of the heterologous group (11). Children previously infected with a virus of one group appear to be somewhat less susceptible to repeat infections with a virus of the same group (20, 36). Studies with RS virus proteins expressed from vaccina virus recombinants suggest that differences in the G protein are responsible for differences in cross protection between the two groups (16, 28, 29). In addition to the differences which occur between the two groups, there are also differences among strains within groups. These differences have been identified by MAb reaction patterns against the G protein (1–3, 10, 12, 13, 19, 21, 26, 33, 35), RNase protection assays on the G protein gene (8, 27), restriction mapping of N gene polymerase chain reaction (PCR) products, partial sequence analysis of the SH gene (5), and nucleotide sequence studies of the G gene (4, 31). Antigenic and genetic studies have each provided important insights into the differences among individual RS viruses.

The greatest differences between the two RS virus groups are seen on the G protein. These differences appear to be important in the host response to infection and are likely to be important in vaccine development. For the purposes of vaccine design, the importance of variations among strains in the same group has not yet been determined. The ability to identify strain differences within groups has, however, provided important information about the epidemiology and transmission of RS virus (2, 25). In this report we describe a practical approach to the characterization of genetic differences among RS viruses on the basis of PCR for amplification of DNA corresponding to parts of the RS virus G and F genes and restriction digests of the PCR products. This assay not only discriminates between the two major RS virus groups but also provides an assessment of genetic variability within the individual groups.

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MATERIALS AND METHODS

Cells and viruses. Forty-seven RS virus isolates were grown in HEp-2 cells in Medium 199 (GIBCO/BRL, Life

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Technologies, Inc.) supplemented with 5% fetal bovine serum with penicillin, kanamycin, and streptomycin. The majority of these isolates have been described previously (30, 31). Additional isolates (WV2780, WV5222, WV6973, WV12342) were generously provided by Maurice Mufson (Huntington, W.V.).

EIA. A group of MAbs reactive with distinct epitopes on the F and G proteins were used to analyze the RS viruses by enzyme immunoassay (EIA). The MAbs, the performance of the EIA, and the subgroup classification scheme were described previously (2). Briefly, each strain was reacted against seven RS virus specific MAbs, four against the G protein and three against the F protein, and one adenovirus-specific biotinylated MAb in a capture EIA. Immulon-2 96-well microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated with a broadly reactive G or F monoclonal capture antibody. Each strain was then reacted with the capture antibody, the panel of eight biotinylated MAbs, peroxidase-conjugated streptavidin, and substrate. A reaction was considered positive if the P/N value was >1.7 and the $P - N$ value was >0.05, where P is the absorbance of the isolate against the MAb. We used two values for N for each strain, one for the MAb against HEp-2 cells and one for the virus strain against the adenovirus MAb. The subgroup and group patterns were then assigned on the basis of the positive and negative reaction patterns described previously (2).

Oligonucleotides. The oligonucleotides used for PCR amplification were based on the published sequences of the G and F genes of the group A and B RS viruses (7, 15, 17, 29, 37). The 3′ oligonucleotide F164 was complementary to bases 164 to 186 in the F protein mRNA of CH18537 (a group B virus) and had one mismatch to the A2 (a group A virus) F protein mRNA sequence (GTTATGACACTGGTATAC CAACC; the mismatch site is underlined) (15). We previously demonstrated that two group-specific oligonucleotides that were complementary to viral G protein mRNA would anneal in a nucleic acid hybridization assay only to A or B group viruses (32). The two 5′ oligonucleotides used for PCR amplification were complementary to these oligonucleotides and conferred group specificity to the PCR amplification. The B group 5′ primer G32 corresponds to bases 10 to 30 in the 8/60 RS virus G protein mRNA (GCAACCATGTC CAAACACAAG) (29). The A group 5′ primer G267 corresponds to bases 247 to 267 in the A2 RS virus G protein mRNA (GATGCAACAAGCCAGATCAAG) (37). The expected size of the PCR product for group A strains is 0.9 kb, and that for group B strains is 1.1 kb.

RNA template preparation. The viral RNA to be used as a template for cDNA synthesis was prepared by a hot phenol extraction procedure (24). Subconfluent monolayers of HEp-2 cells in 35-mm dishes were infected with RS virus. When an extensive cytopathic effect was evident, the cells were scraped into the medium and transferred to a 1.5-ml polypropylene tube on ice. The tube was centrifuged at top speed in an Eppendorf centrifuge (Brinkman Instruments, Inc.) for 2 min at 4°C. The medium was aspirated, the cell pellet was resuspended in 1 ml of cold phosphate-buffered saline and centrifuged for 1 min at 4°C, and the liquid was aspirated. The pellet was resuspended in 0.4 ml of 20 mM sodium acetate–1 mM EDTA (pH 8)–0.5% sodium dodecyl sulfate and frozen at −20°C. The sample was then thawed and mixed by vortex agitation, and 0.4 ml of liquefied phenol was added. The tube was shaken for 4 min in a 45 to 55°C water bath, cooled briefly on ice, and centrifuged for 5 min at room temperature. The aqueous phase was transferred to a new tube and extraction with chloroform-isoamyl alcohol was performed; the aqueous phase was made to 0.1 M with NaCl and was precipitated with ethanol. Immediately before use the sample was centrifuged at 14,000 × g for 20 min at 4°C, and the pellet was washed with 70% ethanol and dried in a Speedvac concentrator (Savant Instruments). The dried pellet was resuspended in 20 μl of H_2O, and 5 μl was used in a first-strand cDNA synthesis reaction. The remaining sample was reprecipitated in ethanol and stored at −20°C.

First-strand cDNA synthesis and amplification. Viral RNA prepared from infected cell lysates as described above was used as a template for cDNA synthesis. A 25-μl reaction volume contained 5 μl of cell lysate RNA, 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL, Life Technologies, Inc.), 20 U of RNase inhibitor (RNasin; Promega, Madison, Wis.), 20 pmol of primer F164 in 0.8 mM deoxynucleoside triphosphates (dGTP, dATP, dTTP, and dCTP; Pharmacia, Inc., Piscataway, N.J.), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. Synthesis was performed for 45 min at 37°C (9). For amplification by PCR (23), the following were added: 50 pmol each of primers F164, G32, and G267 and 2.5 U of *Taq* polymerase (Promega) in 75 μl of buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.8], 1.5 mM $MgCl_2$, 0.1% Triton X-100; supplied by Promega). The sample was covered with mineral oil, and thermal cycling was performed in a thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles and then 72°C for 7 min; the sample was then held at 4°C. A 5-μl volume of each reaction mixture was analyzed by electrophoresis in a 0.7% agarose gel, and the DNA was visualized by ethidium bromide staining. Annealing temperatures needed to be decreased to improve the yields from some strains (see below). If additional DNA was needed, a reamplification was performed with the initial double-stranded DNA as a template. The samples were cleaned by spun column chromatography (Sephacryl S400HR [Sigma] or Chromaspin 400 [Clontech Laboratories Inc., Palo Alto, Calif.]). Sephacryl S400HR has a DNA exclusion limit of 271 bp, and Chromaspin 400 removes fragments of less than 400 bp. Thus, smaller extraneous PCR products were removed, and the samples were precipitated with ethanol, dried, and resuspended in 100 μl of 10 mM Tris-HCl (pH 8)–1 mM EDTA.

Analysis of PCR products. First-strand synthesis from primer F164 yielded a cDNA that extended into the G RNA sequence when the primer annealed to either a polytranscript or to a positive-sense replicative intermediate viral RNA (6). The group-specific 5′ oligonucleotides annealed at different sites in the G protein cDNA sequence. The A and B group G gene-derived PCR products were of different lengths, 676 bases for the A group PCR products and 917 bases for the B group PCR products. The group A products spanned the region coding for most of the G protein ectodomain and included the variable regions described by Cane et al. (4) for the A group G proteins. The group B products included the regions coding for the cytoplasmic tail, transmembrane domain, and the entire ectodomain, and thus included the variable regions of the B group G proteins (31). Additional PCR product length was derived from the intergenic region (52 nucleotides) and part of the F gene (186 nucleotides). Thus, the DNAs encompassed various lengths of the G cDNA and then the intergenic and partial F cDNAs of the same length (14, 15). The products of the B viruses were ≈1.1 kb in length and those of the A viruses were ≈0.9 kb in length (Fig. 1). Agarose gel electrophoresis allowed a

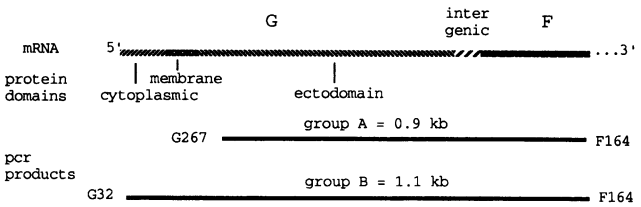

FIG. 1. Locations of primers and sizes of amplified DNAs. A linear representation of a positive-sense RNA transcript corresponding to the region of amplification is shown. The G protein (G), intergenic, and partial F protein (F) RNAs and the major domains of the G protein are indicated. The relative locations of the group A-specific (G267), group B-specific (G32), and group cross-reactive (F164) oligonucleotides are shown, and the product lengths (in kilobases of DNA) are shown.

group designation to be made on the basis of the size of the DNA fragment.

The PCR products (10 to 20 μl) were subjected to restriction endonuclease digestion and then agarose gel electrophoresis to assess the genetic variability within each group. The restriction endonucleases were chosen after analysis of both A and B group RS virus G gene nucleotide sequences (17, 31, 31a, 37). Enzymes which cut once or only a few times in the cDNA were chosen; the enzymes were readily available commercially. For each antigenic group and each enzyme, designations of the different restriction fragment patterns were made.

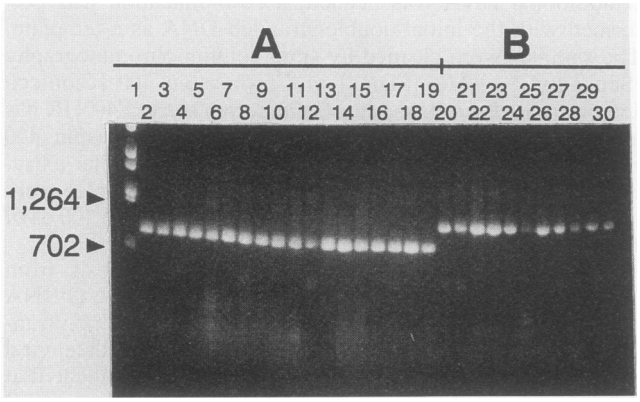

FIG. 2. Discrimination of RS virus antigenic groups by subgroup specific PCR. First-strand cDNAs were amplified by PCR with a common 3′ primer (F164) and by including two potential 5′ primers, G267, which is group A specific, and G32, which is group B specific, using an annealing temperature of 60°C. The products were cleaned by spun column chromatography and were analyzed by electrophoresis in a 0.7% agarose gel, with an ethidium bromide-stained gel shown. Lane 1 is a bacteriophage λ DNA *Bst*EII digestion size marker, with the sizes (in base pairs) shown to left of the gel. The solid line across the top indicates the two antigenic groups. The lane numbers and designations of the group A viruses are as follows: 2, A2; 3, 2139-4; 4, 2045-4; 5, 2045-12; 6, 2045-16; 7, 2045-33; 8, 2045-32; 9, 2045-25; 10, 1836; 11, 2012-8; 12, A1; 13, 1935; 14, 159/59; 15, 2040-35; 16, WV5222; 17, WV2780; 18, WV12342; 19, WV6973. The lane numbers and designations of the group B viruses are as follows: 20, 9320; 21, 2045-4; 22, 2040-23; 23, CH18537; 24, 1776; 25, 1842; 26, 2040-1; 27, 8/60; 28, 1355; 29, WV15291; 30, WV4843.

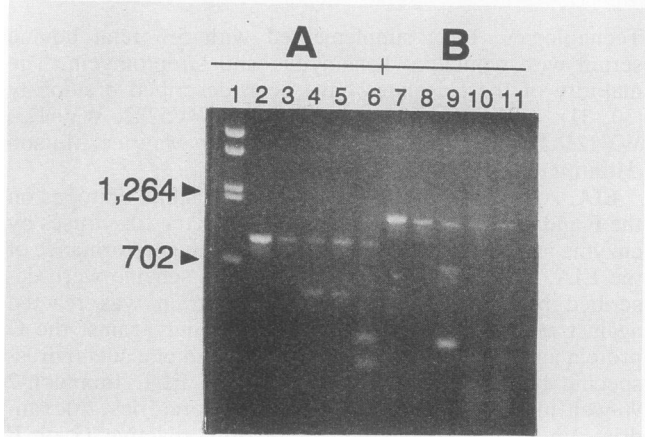

FIG. 3. Discrimination of RS virus antigenic groups by subgroup-specific PCR. Amplification of first-strand cDNAs was performed as described in the legend to Fig. 2; however, the PCR annealing temperatures were decreased to improve the DNA yield. The A and B group viruses are indicated at the top, and lane 1 is a bacteriophage λ DNA *Bst*EII digest size marker, with sizes (in base pairs) indicated to the left of the gel. The lanes, virus designations, and annealing temperatures for the group A viruses were as follows: 2, A2, 60°C; 3, 9324, 40° (lanes 4 to 6 represent 30°C annealing temperature controls, including viruses which were successfully amplified at 60°C); 4, A2, 30°C; 5, 2045-12, 30°C; 6, 2045-25, 30°C. The lanes, virus designations and annealing temperatures for the group B viruses were as follows: 7, 8/60, 60°C; 8, 2045-41, 40°C; 9, 2045-5, 30°C; 10, 2040-21, 30°C; and 11, WV10010, 30°C.

RESULTS

PCR group classification. The PCR product size determined by gel electrophoresis distinguished all 19 antigenic group A isolates from the 15 antigenic group B isolates (Fig. 2 and 3). These isolates demonstrated the expected size characteristics of 0.9 kb for A group viruses and 1.1 kb for B group viruses (Fig. 1). Five viruses (9324, 2045-41, 2045-5, 2040-21, and WV10010) yielded inadequate products at the initial 60°C annealing temperature but produced satisfactory products of the expected size when lower annealing temperatures were used (Fig. 3). Extraneous smaller bands were present at the lower temperatures. These extraneous bands might have interfered with the analysis of the restriction fragments described below. However, they were limited in number and were easily discounted when making restriction fragment comparisons. Uninfected cell extracts processed in an identical manner produced no amplification products.

Restriction endonuclease digestion of amplified DNAs revealed genetic heterogeneity. The PCR-amplified DNAs were subjected to restriction endonuclease cleavage, and the resulting fragments were analyzed by agarose gel electrophoresis. After analysis of each of the isolates (Tables 1 and 2), composite figures were constructed to provide examples of the restriction patterns observed (Fig. 4 and 5). Since the PCR products were of different sizes and the sequences varied extensively for the G gene between the two groups, the restriction fragment patterns were different for the A and the B group viruses. We assigned a lowercase letter to each reaction pattern for the different enzymes to simplify comparisons among isolates. We used the first half of the alphabet for the group A patterns and the second half of the alphabet (beginning with the letter *n*) for the group B viruses. The letters assigned within each restriction digestion are independent of the letters assigned to the other digestions,

TABLE 1. Date and place of isolation of group A RS viruses and demonstration of antigenic and genetic heterogeneities

Virus	Isolation		Subgroup	Restriction patterns within group A[a]			
	Year	Place		*Alu*I	*Pst*I	*Rsa*I	*Hin*cII
2045-16	1984–1985	Minnesota	A/1	a	c	a	b
2045-25	1985–1986	Minnesota	A/1	a	c	a	b
2045-33	1985–1986	Minnesota	A/1	a	a	a	b
1836a	1985	Georgia	A/1	a	c	a	b
159/59	1959	Sweden	A/2	d	a	d	c
9324	1977	Massachusetts	A/2	c	b	c	a
WV2780	1979	West Virginia	A/2	c	b	a	a
2045-1	1984–1985	Minnesota	A/2	b	b	a	a
2045-32	1985–1986	Minnesota	A/2	c	b	b	a
2045-12	1984–1985	Minnesota	A/3	c	b	b	a
2012-8	1986	West Virginia	A/3	a	a	a	a
WV5222	1981	West Virginia	A/4	e	c	b	a
WV12342	1984	West Virginia	A/4	f	a	e	a
1935a	1985	Washington State	A/4	a	c	a	b
A/1	1961	Australia	A/5	a	a	a	a
A/2	1961	Australia	A/5	a	a	a	a
2045-35	1985	Washington State	A/6	a	c	a	a
2139-4	?	West Virginia	A/7	a	b	a	a
WV6973	1982	West Virginia	A/7	a	b	a	a

[a] Restriction patterns among the individual A group viruses as determined from Fig. 4 and as described in the text. The subgroup classification is described in the text.

except that the letter a represents the prototype A2 strain and the letter n represents the prototype 8/60 strain for each digestion. The group A viruses (Fig. 4) yielded six patterns after digestion with *Alu*I, three patterns after digestion with *Pst*I, five patterns after digestion with *Rsa*I, and three distinct patterns after digestion with *Hin*cII. The group B viruses (Fig. 5) produced four patterns after digestion with *Alu*I, four patterns after digestion with *Rsa*I, and two patterns after digestion with *Hin*cII but were not cleaved by *Pst*I (data not shown).

The restriction fragment patterns observed in the present study agreed with the restriction fragment patterns predicted from analysis of the A2 RS virus G gene, intergenic region, and F gene nucleotide sequences (14, 15, 37). The enzymes and the predicted A2 fragments were as follows: *Alu*I, 800, 58, and 57 bp; *Pst*I, 494, 299, and 122 bp; *Rsa*I, 293, 281, 179, and 162 bp; and *Hin*cII, 775 and 140 bp. Fragments of these approximate sizes were observed (Fig. 4), although the smaller fragments were less well visualized. The G gene nucleotide sequences have been determined for the group A viruses WV2780 and WV5222 (31a). The restriction fragment sizes from the G gene-derived sequences were as expected for these isolates. Nucleotide sequence variability within the intergenic and F gene regions of these isolates was revealed by changes in the lengths of fragments which, by comparison with the A2 fragments, originated from the intergenic and F gene regions.

The predicted restriction fragment sizes may be compared for the group B isolates. The complete G gene, intergenic, and F gene sequences are available for the 18537 virus (14, 15, 17). The restriction enzymes and the predicted fragments for 18537 were as follows: *Alu*I, 833 and 322 bp; *Pst*I, did not cut; *Rsa*I, 1,043 and 112 bp; and *Hin*cII, 852 and 303 bp. Except for *Rsa*I, the patterns were identical for 18537 and

TABLE 2. Date and place of isolation of group B RS viruses and demonstration of antigenic and genetic heterogeneities

Virus	Isolation		Subgroup	Restriction patterns within group B[a]			
	Year	Place		*Alu*I	*Pst*I	*Rsa*I	*Hin*cII
8/60	1960	Sweden	B/1	n	n	n	n
9320	1977	Massachusetts	B/1	o	n	o	o
1776	1984	Georgia	B/1	o	n	o	o
1842	1985	Georgia	B/1	n	n	p	o
2040-23	1985	Washington State	B/1	q	n	q	o
WV4843	1980–1981	West Virginia	B/2	o	n	o	o
WV10010	1983	West Virginia	B/2	q	n	o	o
2045-4	1984–1985	Minnesota	B/2	p	n	o	o
2045-5	1984–1985	Minnesota	B/2	n	n	p	o
2045-41	1985–1986	Minnesota	B/2	o	n	o	o
2040-1	1985	Washington State	B/2	o	n	o	o
2040-21	1985	Washington State	B/2	q	n	q	o
NM1355	1989	New Mexico	B/2	p	n	o	o
18537	1962	District of Columbia	B/3	n	n	o	n
WV15291	1985	West Virginia	B/3	q	n	o	o

[a] Restriction patterns among the individual B group viruses as determined from Fig. 5 and as described in the text. The subgroup classification is described in the text.

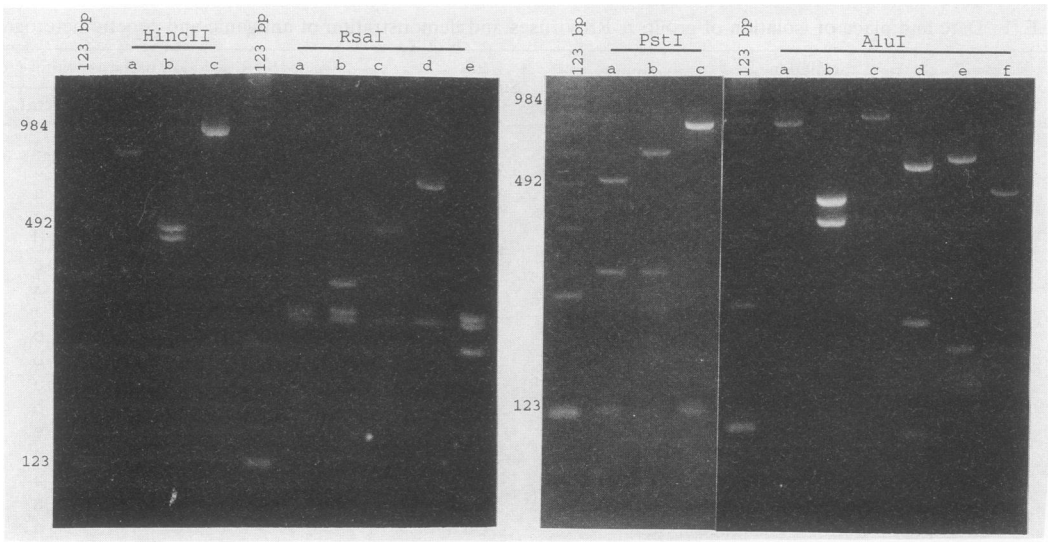

FIG. 4. Restriction fragment analysis of amplified DNAs of group A viruses. The PCR products of the viruses listed in Table 1 were each digested with *Alu*I, *Pst*I, *Rsa*I, and *Hin*cII and were separated by agarose gel electrophoresis. Each different restriction pattern was assigned a lowercase letter designation, and examples of each of these patterns are shown after electrophoresis in a 3% agarose gel. The first lane in each section is a 123-bp size marker (GIBCO/BRL, Life Technologies, Inc.), the restriction endonucleases, the assigned lowercase letters, and viruses are as follows: for *Hin*cII, a, A2; b, 1836; and c, 159/59; for *Rsa*I, a, A2; b, WV5222; c, 9324; d, 159/59; and e, 12342; for *Pst*I, a A2; b, 9324; and c, WV5222; and for *Alu*I, a, A2; b, 2045-1; c, 9324; d, 159/59; e, WV5222, and f, 12342.

8/60; after the *Rsa*I digestion, the largest 8/60 fragment was smaller than the 18537 fragment, which is in agreement with the existence of a *Rsa*I site in the 8/60 but not the 18537 G gene cDNA sequence. The G gene sequences have also been determined for the group B viruses 9320, WV4843, WV10010, WV15291, and NM1355 (31). The fragment sizes agreed with the predicted fragments on the basis of these sequences. Like for the group A viruses, some group B virus restriction patterns revealed changes in the lengths of fragments derived from the intergenic and F gene regions. The sequences described above also confirmed that the differences between the *Alu*I and *Rsa*I n and o patterns (for the 8/60 and 9320 viruses, respectively) were correct. The differences among these large fragments were better appreciated on lower-concentration agarose gels (data not shown) than on the 3% agarose gels used for Fig. 5. The band slightly larger than 246 bp in the *Hin*cII lane (lane o, Fig. 5) was present in the original PCR product and was not a restriction digestion product.

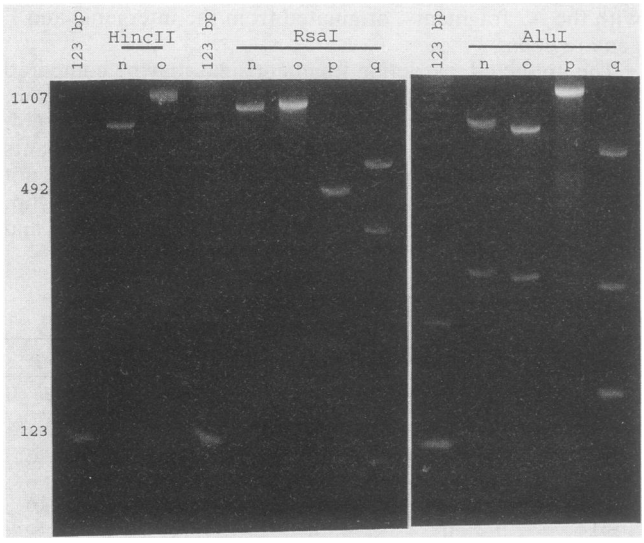

FIG. 5. Restriction fragment analysis of amplified DNAs of group B viruses. The PCR products of the viruses listed in Table 2 were each digested with *Alu*I, *Pst*I, *Rsa*I, and *Hin*cII and were separated by agarose gel electrophoresis. Each different restriction pattern was assigned a lowercase letter designation, and examples of each of these patterns are shown after electrophoresis in a 3% agarose gel. The *Pst*I digestion is not shown because it did not cleave the group B products. The first lane in each section is a 123-bp size marker; the restriction endonucleases, the assigned lowercase letters, and viruses are as follows: for *Hin*cII, n, 8/60, and o, 2040-23; for *Rsa*I n, 8/60; o, 1776; p, 1842; and q, 2040-23; and for *Alu*I n, 8/60; o, 9320; p, 2045-4; and q, 2040-23.

The restriction patterns for the group A and B viruses are summarized in Tables 1 and 2. Most of the viruses from the same antigenic subgroup gave different restriction patterns. The number of restriction patterns among each subgroup was as follows. The four A/1 viruses had two restriction patterns, the five A/2 viruses had five, the two A/3 viruses had two, the three A/4 viruses had three, the two A/5 viruses had one, the one A/6 virus had one, and the two A/7 viruses had one; there was only one A/6 virus. The five B/1 viruses had four restriction patterns, the eight B/2 viruses had five, and the two B/3 viruses had two. In a few instances, the MAb reaction patterns revealed differences that were not seen with the restriction patterns. For example, the group A viruses 1836a (subgroup A/1) and 1935a (subgroup A/4) had the same restriction patterns, and the group B viruses 2040-23 (subgroup B/1) and 2040-21 (subgroup B/2) had the same restriction patterns.

Antigenically similar isolates from a single epidemic are genetically heterogeneous. Thirteen group A RS viruses isolated during a single epidemic period (1989) at the University of New Mexico Hospital were also analyzed (30). Twelve isolates were antigenic subgroup A/2 and one was antigenic

TABLE 3. RS viruses from New Mexico[a]

Virus	Subgroup	Restriction pattern[b]	
		*Taq*I	*Ava*II
A2	A/5	a	a
89-937	A/2	a	b
89-1103	A/2	a	b
89-4928	A/2	a	b
89-1249	A/2	a	b
89-804	A/2	a	b
89-318	A/4	a	a
89-743	A/2	a	b
89-1243	A/2	a	b
89-1325	A/2	b	a
89-1226	A/2	b	a
89-384	A/2	a	b
89-1051	A/2	a	b
89-1006	A/2	a	b

[a] Group A viruses from New Mexico that were isolated in 1989 (30), with the prototype A2 virus included as a control.
[b] Restriction pattern designations were assigned as described in the text. The subgroup classification is described in the text.

subgroup A/4. Thus, the 12 A/2 subgroup viruses were antigenically identical in their reactivities with the MAbs tested. All 13 viruses yielded the expected 0.9-kb fragment upon group-specific PCR amplification, with 10 isolates producing the best products after amplification with an annealing temperature of 30°C (data not shown). The amplified DNAs were subjected to restriction endonuclease cleavage (data not shown); the results are summarized in Table 3. The one A/4 isolate was distinct from the 13 A/2 isolates. Although digestion with *Hin*cII, *Rsa*I, *Pst*I, and *Alu*I did not discriminate among the A/2 isolates, two other enzymes, *Taq*I and *Ava*II, did. With these two enzymes, two restriction patterns were seen, one for two isolates (89-1325 and 89-1226) and a second for the other 10 A/2 subgroup viruses. Thus, even in the population of antigenically similar (subgroup A/2) viruses from a single epidemic described here, restriction fragment differences were noted.

DISCUSSION

We described a new approach to the analysis of individual RS virus isolates. The approach discriminates between the two major antigenic groups and can be used to discriminate genetic variants within each group. The viral RNA present in infected cell lysates was used as a template for first-strand cDNA synthesis from a 3′ primer that annealed to the RNAs of both group A and group B viruses. Additional 5′ primers were added; they were A or B group specific and annealed at different sites in the cDNA molecule. PCR amplification resulted in DNA products of different sizes that were easily discriminated by agarose gel electrophoresis. All 47 viruses tested (Tables 1, 2, and 3) were given the same group classification as that determined with a previously described MAb-based system (3). Since the viruses tested represented multiple antigenic subgroups and were temporally and geographically diverse in their origins, it is likely that this system will be generally applicable for grouping RS virus strains.

MAb-based assays have been the standard for the group classification of RS virus isolates (3, 21). Recently, other systems have been described, including nucleic acid hybridization with group-specific G gene cDNA or synthetic oligonucleotide probes (30, 32) and P protein mobility analysis (34). Cane and Pringle (5) have also described a PCR-based approach that discriminates the two RS virus groups on the basis of differential amplification of the SH genes. The direct amplification of RS virus F gene products from clinical samples has recently been described (22), suggesting that our assay might be applied directly to clinical samples.

The degree of genetic variability occurring among the viruses that we tested was examined by restriction endonuclease cleavage of the PCR-amplified DNAs and analysis of the cleavage fragments by agarose gel electrophoresis. Interestingly, we demonstrated extensive variability among viruses of the same antigenic group and subgroup (Tables 1 and 2). In the present study, most of the temporally and geographically diverse isolates with identical MAb reaction patterns had different restriction patterns. This is consistent with previous studies which demonstrated greater differences with genetic characterization than with antigenic characterization (25). Also, there was antigenic variability among isolates with identical restriction patterns, indicating additional genetic variability beyond that detected by the use of four restriction enzymes. Among the 12 A/2 antigenic subgroup viruses isolated during a single epidemic period in New Mexico, different restriction patterns were also observed (Table 3). Thus, even among these viruses of the same antigenic subgroup from the same outbreak, we identified two different restriction patterns. This indicates that the MAb reaction patterns gave only a limited picture of the diversity among these isolates. As suggested previously (25), the MAb reaction patterns should be used only to infer the similarity among isolates from the same outbreak, and even then genetic studies are needed to confirm similarities among isolates.

Since the existence of two major antigenic groups of RS virus was described (3, 21), several investigations have demonstrated that within each group additional variation occurs. Such variability has been described by using MAb reactivity (1–3, 10, 12, 13, 19, 21, 26, 33, 35), RNase protection assays (8, 27), nucleotide sequence analysis (4, 5, 17, 29, 31), and restriction enzyme cleavage of PCR amplification products (5). The RNase protection assays have advanced our understanding of RS virus epidemiology by demonstrating genetic differences among antigenically and epidemiologically similar viruses (25). However, the RNase protection assays are somewhat restricted in their applicability because of their use of radioisotopes. The RNase protection assays use cDNA probes that originated from prototype group A viruses and measure the differences relative to those sequences. These probes potentially underestimate the differences that occur among the viruses being examined, particularly the group B viruses. Nucleotide sequence analysis provides detailed information, but it is too labor intensive to be broadly applicable.

The SH and N genes of RS virus have also been analyzed (5). PCR-amplified N gene products were analyzed by restriction fragment electrophoresis, and viruses from a single epidemic period showed two patterns among the A group isolates and two patterns among the B group isolates. For the SH gene, nucleotide sequence analysis was performed, and four separate lineages among the group A viruses from a single epidemic period were described. The amplification products that we used included part or all of the G gene, the intergenic region, and part of the F gene including the region coding for the F protein signal peptide. The G gene varies between and within groups, and the intergenic and F gene regions also vary (14, 15). Thus, the amplification products that we used may be expected to maximize the opportunity

to recognize genetic variability among the viruses. However, since the assay that we described provides information about only a fraction of the nucleotides in the amplification products, similarities among viruses will need to be confirmed by additional study.

Here we reported the use of PCR for the group classification of RS viruses. Importantly, the restriction fragment analysis of the PCR products provided a facile means of screening for genetic variation among viruses of the same group. The assay that we described provides a new tool for the study of the molecular epidemiology of RS viruses.

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