JOURNAL OF VIROLOGY, Apr. 1990, p. 1869–1872
0022-538X/90/041869-04$02.00/0

Vol. 64, No. 4

Molecular Cloning of Pneumonia Virus of Mice

P. CHAMBERS, J. BARR, C. R. PRINGLE, AND A. J. EASTON*
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

Received 7 August 1989/Accepted 15 December 1989

cDNA clones representing nine genes of pneumonia virus of mice (PVM) have been generated. The sizes of the corresponding mRNAs and a provisional transcriptional map of the virus genome have been determined. The apparent gene order is very similar to that of respiratory syncytial virus. The sequences adjacent to the 3′ termini of the PVM genes were determined and are very similar to those of respiratory syncytial virus. Several PVM gene polypeptide products have been assigned.

Pneumonia virus of mice (PVM) is a member of the *Pneumovirus* genus of the family *Paramyxoviridae*, together with human and bovine respiratory syncytial (RS) viruses (24). Recently it has been proposed that the etiological agent of turkey rhinotracheitis (turkey rhinotracheitis virus) should also be classified in this genus (4, 6, 26). PVM was first isolated from mice and was associated with latent or inapparent infection (22, 23). Following passage of lung tissue from infected animals by intranasal inoculation, recipient mice develop fatal pneumonia (22, 23). PVM is common in most colonies of laboratory mice (1, 15, 30). Antigenically related viruses have been isolated from other rodents (14, 20), and serological evidence has indicated that an antigenically related virus(es) can infect many other species, including humans (20, 21, 23, 31). The age distribution of seropositive children and adults resembled that observed with RS virus, but no disease has yet been correlated with seroconversion (31).

PVM is serologically distinct from the RS viruses, but the nucleocapsid proteins of PVM and human RS virus are antigenically related (16, 28). In common with RS virus, PVM encodes more polypeptides than the six or seven major gene products of other paramyxoviruses (2, 3, 27, 28). The genome of RS virus is a single-stranded negative-sense RNA molecule of approximately 15,300 nucleotides, which encodes 10 unique genes.

cDNA clones were generated by using cytoplasmic RNA from PVM-infected, dactinomycin-treated BSC-1 cells (17, 25). Virus-specific clones were identified by the ability to hybridize with ^{32}P-labeled cDNA to RNA from infected cells while showing no hybridization with cDNA generated by using RNA from uninfected cells. A total of approximately 500 potentially PVM-specific clones were separated into nine distinct groups on the basis of extensive analysis by colony hybridization and restriction endonuclease mapping. The relative abundance of cDNA clones representing each of these groups ranged from 18% of all potential virus-specific clones to 2% (Fig. 1B). Representative clones from each group were screened by hybridization to Northern (RNA) blots of RNA from PVM-infected cells to identify the mRNA from which each was derived (29; Fig. 1A). cDNA clones from each group hybridized to a major mRNA species, and most groups hybridized to minor species, presumably polycistronic readthrough transcripts, as described for many negative-strand viruses (11, 13, 18, 19, 34). The estimated sizes of the major mRNA species and polycistronic mRNAs are given in Table 1. The sizes of RS virus mRNAs are given for comparison.

Several cDNA clones representing polycistronic mRNAs were isolated. For example, Fig. 1, lane E, shows the hybridization pattern of a cDNA clone which hybridized to clones of both groups 5 and 6. This clone hybridized to an mRNA of approximately 1,200 nucleotides, to which group 5-specific clones also hybridized, and an mRNA of approximately 500 nucleotides, to which group 6-specific clones also hybridized (data not shown). The third mRNA species, of approximately 1,700 nucleotides, represents the polycistronic RNA observed when using either group-specific probe. The polycistronic RNA of genes 5 and 6 was as abundant on Northern blots as the smaller mRNA (Fig. 1A). This was also reflected in the proportions of the cDNA clones obtained, with the polycistronic species being so abundant that no meaningful estimate of the abundances of the two individual mRNAs could be made. This suggests that the termination of transcription following gene 5 is relatively inefficient, although the reasons for this are unclear. The sequence at the 3′ end of gene 5 is similar to those of the other genes, as described below.

The abundance of cDNA clones in each hybridization group and the detection of polycistronic RNA species permit tentative ordering of genes on the virus genome. Thus, the polycistronic RNA to which group 3 clones hybridize is the same as that observed with group 4 clones and has a size equivalent to the sum of both major mRNAs (Fig. 1A, lanes C and D; Table 1). This indicates that these two genes are adjacent on the genome. The order of the genes was confirmed by sequencing the junction between the two genes in a cDNA clone representing the polycistronic RNA linking the two (data not shown). Similarly, the isolation and sequencing of clones from polycistronic RNA established the linkage and order of genes 1 and 2, 5 and 6 (Fig. 1A, lane E), and 7 and 8 (Fig. 1A, lane F). This is summarized in Fig. 1B.

The relative order in the virus genome of these linked pairs of genes was suggested by considering the relative abundances of the cDNA copies of the mRNA transcripts. We expect that, in general, mRNA transcripts, and hence cDNA clones from genes located near the 3′ terminus of the genome, will be more abundant than those from more distant genes, although differential mRNA stability or extraction and variation in cloning efficiency may distort particular data. Genes 1 and 2 were ordered relative to each other by isolation of a cDNA clone of a polycistronic RNA and, on the basis of abundance, were considered to precede genes 3 and 4, which are unequivocally linked. Genes 5 and 6 were linked and considered to follow 3 and 4 but precede 7 and 8,

* Corresponding author.

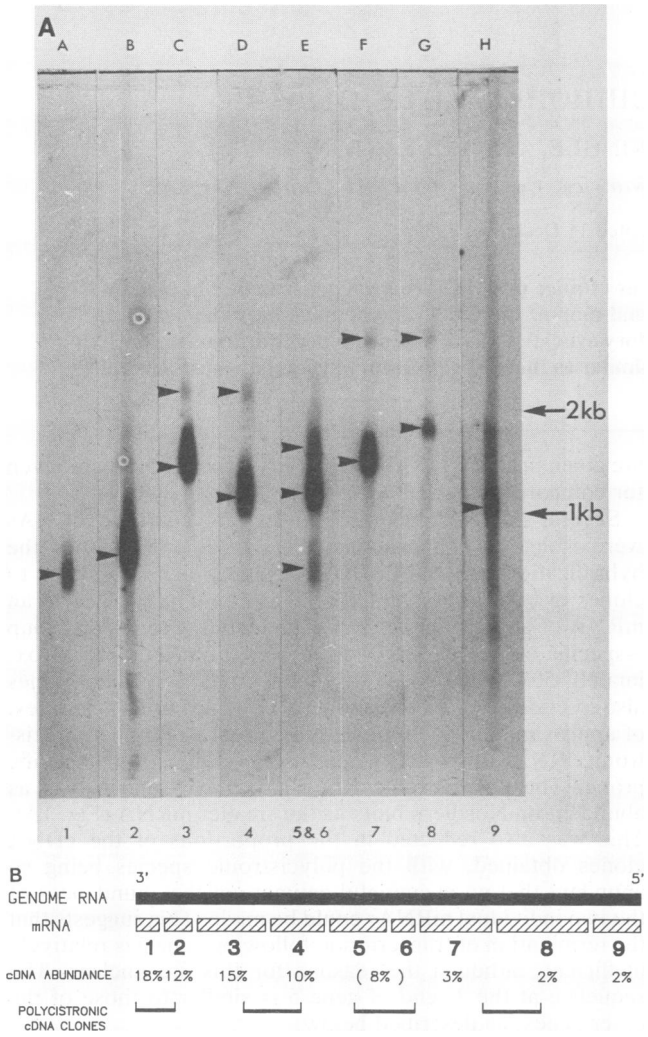

FIG. 1. (A) Autoradiograph of Northern blots of cytoplasmic RNA prepared from PVM-infected BSC-1 cells. Each strip was hybridized individually with ^{32}P-labeled plasmid DNA containing PVM sequences. The numbers at the bottoms of the lanes indicate which virus-specific clone groups were used. Two numbers in lane E indicate that a clone representing a polycistronic mRNA was used. The arrows show the positions of size markers of 1,000 and 2,000 nucleotides. (B) Putative gene order for PVM indicating the relative abundances of cDNA clones from all of the genes. Several polycistronic cDNA clones from genes 5 and 6 were obtained; therefore, data on the abundances of individual cDNAs were not meaningful. Genes which were shown to be linked by isolation of cDNA clones representing polycistronic mRNAs are indicated. The bars below the clone abundances show the locations of polycistronic cDNA clones that link groups 1 and 2, 3 and 4, 5 and 6, and 7 and 8.

which were also linked. The order of genes in linked pairs was determined by characterization of polycistronic cDNAs and nucleotide sequence analysis of the junctions and is consistent in each case with the clone abundance data for these pairs (Fig. 1B). Gene 9 was located following linked genes 7 and 8 solely on the basis of abundance.

No cDNA clones representing a large mRNA capable of encoding the L polypeptide were identified, probably because of the low abundance of this mRNA.

A putative gene order for PVM was derived by using these data (Fig. 1B) and assuming a 3'-proximal location for the most abundantly represented genes. The number of distinct

TABLE 1. Summary of mRNA sizes[a] and polypeptide assignments of the nine groups represented by unique cDNA clones generated from PVM and mRNA sizes[b] and polypeptide assignments of RS virus

Hybridization group	PVM			RS virus		
	Major mRNA species (nucleotides)	Minor RNA species (nucleotides)	Polypeptide (kilodaltons)	Gene	Size (nucleotides)	Polypeptide (kilodaltons)
1	500		14.0	1C	528	14.0
2	600		19.0	1B	499	11.0
3	1,400	2,500	39.0	N	1,197	42.0
4	1,100	2,500	41.0, 37.0	P	907	34.0
5	1,200	1,700	24.0	M	952	26.0
6	500	1,700		1A	405	9.5
7	1,500	3,500		G	918	32.5
8	1,900	3,500	49.0	F	1,899	59.0
9	1,000			22K	957	24.0

[a] Includes the poly(A) tract.
[b] Excludes the poly(A) tract.

genes, the sizes of the corresponding mRNAs, and the gene order suggest that PVM is similar to RS virus and support their assignment to the same genus.

Clones representing the 3' end of each of the nine PVM genes were readily isolated, since the cDNA synthesis was primed by using oligo(dT), and the nucleotide sequences immediately adjacent to the poly(A) tracts were determined (32). These sequences closely resemble each other and are similar to the corresponding sequences of RS virus genes (Fig. 2). Consensus sequences for the 3' ends of PVM and RS virus genes are almost identical, and all genes match TAGTTA in at least five of six positions (8).

Polypeptide products were assigned to PVM genes by hybrid arrest of in vitro translation. DNAs from individual recombinant bacteriophage M13 containing antisense sequences from PVM genes were immobilized on separate nitrocellulose filters, and total cytoplasmic RNA from PVM-infected cells was hybridized to each in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5)–400 mM NaCl at 60°C for 20 min. The unbound RNA was precipitated in ethanol and used to direct the synthesis of polypeptides in vitro. The absence of a polypeptide from the in vitro translation products indicated that it was the product of the gene subcloned into M13. The results of this

PVM		RS virus	
GENE	3'–END SEQUENCE	GENE	3'–END SEQUENCE
1	TAGTTAATT	1C	TAGTTAATAT
2	TAGTTATAG	1B	TAGTAATTT
3	TATTTAATT	N	GAGTTAAT
4	TAGTTAATT	P	TAGTTAC
5	TAGTTAAAT	M	AAGTTAAT
6	TAGTTAAC	1A	TAGTTAATT
7	TAGTTAATG	G	TAGTTACTT
8	TAGTTAATT	F	TAGTTATAT
9	TAGTTATAT	22K	TAGTTATTT
CONSENSUS:	PVM	TAgTTAatt	
	RS virus	tAGTtantt	

FIG. 2. Polyadenylation signal sequences of PVM and RS virus genes. Nucleotide sequences are shown as cDNAs of mRNA sense. Consensus polyadenylation sequences are shown for both viruses.

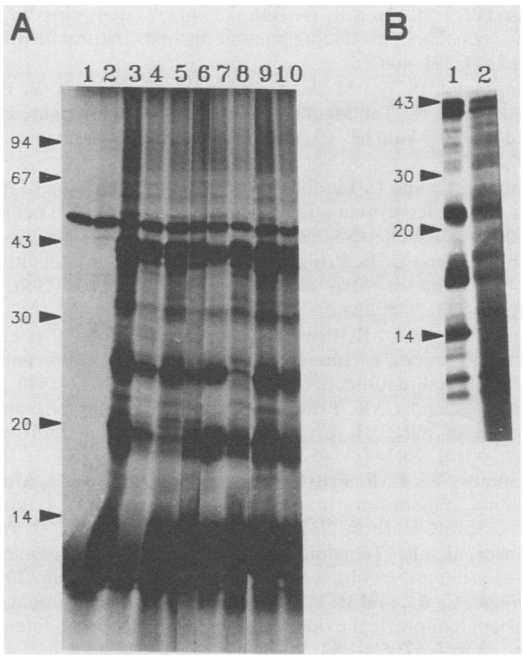

FIG. 3. Hybrid-arrested translation in vitro of PVM-infected cell mRNA by cloned cDNA. (A) Translation in rabbit reticulocyte lysate. Lanes: 1, no RNA; 2, mock-infected BSC-1 cell mRNA; 3, PVM-infected BSC-1 cell mRNA; 4 to 10, PVM-infected cell mRNA following hybridization to filter-bound DNA representing genes 1 to 5, M13 mp18, and gene 8, respectively. (B) Translation in wheat germ extract. PVM-infected cell mRNA following hybridization to filter-bound DNA representing M13 mp18 (lane 1) and gene 1 (lane 2). Arrowheads indicate the positions of molecular size markers (sizes are given in kilodaltons).

analysis are shown in Fig. 3. A summary of the results is given in Table 1, together with the molecular weights of the equivalent RS virus polypeptides. Samples were labeled with [^{35}S]methionine and analyzed on a 10% (wt/vol) polyacrylamide gel that contained 50% (wt/vol) sucrose to improve the resolution of small polypeptides (5).

Six of the nine genes were assigned in this way. Polypeptides corresponding to the remaining three genes were not identified, possibly because of the low abundances of the corresponding mRNAs, low methionine content, or comigration with a major polypeptide, which masked their disappearance. Polypeptide products were assigned for genes 1 to 5 and 8.

Hybridization with cDNA from gene 1 removed a polypeptide with a molecular weight of 14,000 (Fig. 3A, lane 4, and Fig. 3B, lane 2) similar to that of the RS virus 1C gene, which occupies a similar promoter-proximal location (9, 11, 12).

Gene 2 encodes a polypeptide with a molecular weight of 19,000 (Fig. 3A, lane 5), which is markedly different from the size (11,000) of the product of the corresponding 1B gene in RS virus (12).

Gene 3 encodes a polypeptide with a molecular weight of 39,000 (Fig. 3A, lane 6) which is very similar in molecular weight to the N protein of RS virus (7, 9). This polypeptide has been identified previously as the nucleocapsid protein of PVM (3, 16).

Gene 4, which corresponds in the gene order to the phosphoprotein of RS virus, appears to direct the synthesis of two polypeptides with molecular weights of 37,000 and 41,000 (Fig. 3A, lane 7). These two polypeptides, identified as P and 39K by Ling and Pringle (27), who used a different gel system, are immunologically related and share common peptides following limited proteolytic digestion. It seems likely that the two polypeptides represent a single protein which undergoes posttranslational modification or that two in-frame AUG initiation codons are present in the same open reading frame. Sequence analysis of P gene cDNA clones will clarify this situation.

Gene 5 directs the synthesis of a single polypeptide with a molecular weight of 24,000 (Fig. 3A, lane 8), previously designated as the PVM M polypeptide, which corresponds closely to the RS virus M protein (9, 27, 33).

Gene 8, the putative gene encoding the fusion (F) protein, directs the synthesis of an unglycosylated polypeptide with an apparent molecular weight of 49,000 (Fig. 3A, lane 10). This size is somewhat smaller than the unglycosylated molecular weight (59,000) of the RS virus F protein (10).

The remaining three PVM genes, 6, 7, and 9, which have not had polypeptide products assigned, correspond in the proposed PVM gene order to the 1A, G, and 22K genes, respectively, of RS virus.

The data presented here suggest that the gene order of nine PVM genes, excluding L, on the virus genomic RNA is similar to that of RS virus and assign polypeptide products to six of these. These data suggest a close evolutionary relationship between RS virus and PVM, quite distinct from other paramyxoviruses. PVM may therefore provide a good model system, in the most convenient small animal as a natural host, for analyzing the immunology and pathogenesis of pneumovirus infection.

This work was funded by Medical Research Council grant G8514410 CA and Wellcome Trust grant 15847/1.5.

We thank Roger Ling for PVM-infected cell RNA and Claire Halpin for wheat germ extract.

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