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Defective Interfering Virus Associated with A/Chicken/Pennsylvania/83 Influenza Virus

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The A/Chicken/Pennsylvania/1/83 influenza virus, isolated from a respiratory infection of chickens, is an avirulent H5N2 virus containing subgenomic RNAs (W. J. Bean, Y. Kawaoka, J. M. Wood, J. E. Pearson, and R. G. Webster, J. Virol. 54:151–160, 1985). We show here that defective interfering particles are present in this virus population. The virus had a low ratio of plaque-forming to hemagglutinating units and produced interference with standard virus multiplication in infectious center reduction assays. Subgenomic RNAs were identified as internally deleted polymerase RNAs. We have confirmed that this virus protects chickens from lethal H5N2 influenza virus infection. This protective effect appeared to be due to the inhibition of virulent virus multiplication. Additionally, subgenomic RNAs derived from polymerase RNAs were detected in 5 of 18 RNA preparations from animal influenza virus isolates. Therefore, defective interfering particles are sometimes produced in natural influenza virus infections, not just under laboratory conditions. These particles may be capable of suppressing the pathogenic effect of virulent virus infections in nature.

Defective interfering (DI) particles of influenza virus contain subgenomic RNAs which are standard gene segments with internal deletions (for a review, see reference 25). The replication of DI particles depends upon helper functions provided by standard influenza virus. DI particles interfere with the replication of the helper virus and can modify standard viral pathogenesis in experimental animals (for a review, see references 2 and 25). Depending on the virus/host system and dosage used, DI particles may protect a host from lethal infection (16, 27, 30), prolong the course of the disease (17), or convert the acute infection to a persistent state (14). DI particles are easily generated under laboratory conditions. However, whether DI particles occur in natural influenza virus infections and whether they can modulate the pathogenicity of virus outbreaks, as suggested by Huang and Baltimore (18), are unanswered questions.

In April 1983, an H5N2 influenza virus outbreak occurred with low mortality in poultry in the eastern United States. The virus first isolated from this outbreak, A/Chicken/Pennsylvania/1/83 (CP1), was avirulent, and RNA preparations from this virus contained subgenomic RNAs (3). In October 1983, a closely related but highly virulent virus, A/Chicken/Pennsylvania/1370/83 (CP1370), emerged and spread to domestic poultry in four states, causing up to 80% mortality (23). CP1370 RNA preparations did not contain subgenomic RNAs. In experimental mixed infections, CP1 protected chickens from the pathogenic effect of CP1370 (3). Therefore, it is possible that DI particles associated with CP1 played a role in controlling mortality from the H5N2 virus outbreak in mid-1983, until CP1370 became dominant (3). If so, this would be the first reported instance of the involvement of DI particles in a natural influenza virus infection.

The experiments in this paper were done to determine whether CP1 contained DI particles, based on analyses of defectiveness, interference with standard virus multiplication, and the primary structure of subgenomic RNAs. Additionally, by screening influenza virus isolates derived from natural sources for subgenomic RNAs, we sought to determine whether DI particles are commonly found in natural influenza virus infections. Our results indicate that CP1 contained naturally generated DI particles and that some other influenza virus isolates contained subgenomic RNAs like those of DI particles.

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MATERIALS AND METHODS

Viruses and viral RNA. The avirulent CP1 virus (designated 83-21525) was obtained in allantoic fluid from the National Veterinary Services Laboratory, Ames, Iowa. A clonal isolate was obtained by twice-repeated limit-dilution passage in 11-day-old embryonated chicken eggs (23), followed by two egg passages (1:100 dilution) to produce virus stocks for these experiments. To ensure that these manipulations were not responsible for generating the subgenomic RNAs, we obtained the original material from infected chickens from Robert Eckroade, University of Pennsylvania, Philadelphia, and passaged it once in eggs at high dilution. Viral RNA prepared from the last egg passage appeared the same on polyacrylamide gels as did RNA prepared from the first egg passage of the original swabs from chickens.

The virulent CP1370 virus was also obtained in allantoic fluid from the National Veterinary Services Laboratory. A clonal isolate was obtained by repeated plaque purification on chicken embryo fibroblast cells (W. Bean, personal communication) and was amplified by three egg passages (1:100 dilution) to produce virus stocks for these experiments. The resulting virus preparation is similar in virulence and RNA pattern to the uncloned CP1370 preparation. All work with infectious Chicken/Pennsylvania viruses was done in a P3 containment facility.

Other viruses (see Table 4) were from the repository of influenza virus isolates at St. Jude Children's Research Hospital and were grown in embryonated eggs. RNAs of these viruses were prepared from the second egg passage (1:100 dilution) of the original sample.

Virus RNAs were prepared as previously described (5). RNAs were analyzed by electrophoresis on 3% polyacrylamide–7 M urea gels followed by staining with a silver stain kit (Bio-Rad Laboratories, Richmond, Calif.).

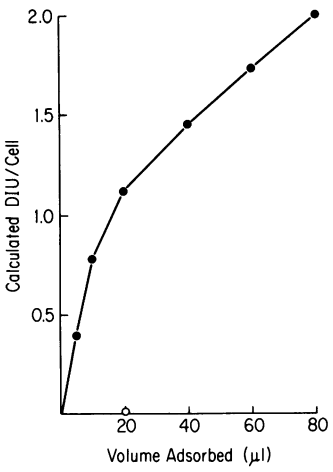

FIG. 1. Infectious center reduction assays were done and analyzed by the method of Janda et al. (20) by using WSN standard virus and chicken embryo fibroblast cells. DIU per cell multiplicity was calculated as follows: $m = -\ln P$, where m equals DIU per cell, and P is the fraction of cells not receiving DI, as determined from infectious center counts (20). Symbols: ●, DI levels of CP1, ○, the DI level of CP1370. A multiplicity of 1.0 is equal to 1.28×10^5 DIU.

For in vitro transcription experiments, viruses were purified, pelleted, and suspended as described (5). Virus protein concentrations (means of four measurements) were determined with a protein assay (Bio-Rad). In vitro transcription reactions were carried out as previously described (13, 26) by using 0.4 mM ApG as the primer. In each reaction, 25 μCi of [α-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) was included to radiolabel the transcription products. Reactions were carried out at 31°C, and aliquots were removed at intervals to assay trichloroacetic acid-insoluble radioactivity.

Interference assays. Infectious center reduction assays for quantitation of DI units (DIU) were performed and analyzed by the method of Janda et al. (20), using chicken embryo fibroblast cells.

For in vivo interference experiments, adult White Leghorn chickens were inoculated with mixed-virus preparations through the nasal cleft. Virus preparations included 10^4 50% egg infective doses (EID_{50}) of CP1370 plus 10^6 EID_{50} of either CP1 or Duck/Michigan/25/80. The two groups of birds were housed in separate cubicles and observed daily for disease signs. Tracheal and cloacal swabs were taken 3 days after inoculation.

Northern blots. Samples of viral RNA were glyoxal denatured, electrophoresed on 1.5% agarose–10 mM sodium phosphate (pH 7.0) gels (29), and then transferred by capillary blotting to GeneScreen Plus hybridization membranes (DuPont-New England Nuclear Research Products, Boston, Mass.). For analysis of CP1 subgenomic RNAs, six equivalent lanes of blotted CP1 RNA were prepared from a single agarose gel. These six blots were repeatedly reused, by elution of one probe and rehybridization with another, to ensure positive identification of bands from the various hybridizations.

The oligonucleotide probes used (see Table 3) matched regions of the published sequences of influenza virus A/NT/60/68 genes (6, 7, 19, 22). Exceptions were HA-44 and NA-146, which correspond to the published CP1 hemagglutinin (HA) and neuraminidase sequences (15, 23), and M-8, based on the A/PR/8/34 sequence (33). Most probes were 12 mers. The probe number indicates the base of the complete gene sequence (plus sense) corresponding to the 5′-terminal base of the probe. Also, a probe matching the universal influenza A RNA 3′ terminus (plus sense) was synthesized. Probes were 5′ end labeled with ^{32}P by using T_4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Unincorporated ^{32}P was removed by using NENSORB 20 cartridges (DuPont-New England Nuclear Research Products).

Blots were prehybridized and hybridized in 6× SSC (1× SSC is 0.15 M NaCl, plus 0.015 M sodium citrate)–10× Denhardt solution–10% dextran sulfate–0.5% sodium dodecyl sulfate (31). The probe concentration was usually 2×10^5 cpm/ml. Hybridizations were carried out at 25°C, except for probes PB1-1375 and NA-146 in which a temperature of 35°C was used to eliminate partial cross-hybridization to other standard gene segments. A modest level of hybridization stringency was used because in most cases we were uncertain of perfect complementarity between the probe and target sequence. Several other probes failed to hybridize to the correct standard gene segment and were not used for analysis. Blots were washed extensively with 6× SSC–0.1% sodium dodecyl sulfate at the hybridization temperature and then sealed without drying into plastic bags for autoradiography. Hybridized probes were eluted from the blots by washing with boiling 0.01× SSC–0.01% sodium dodecyl sulfate, followed by autoradiography to ensure probe removal.

Densitometric quantitation of autoradiographs was done with a Hoefer GS300 scanning densitometer and a computerized data analysis program developed and kindly provided by Michael Ando and Victor Fried of St. Jude Children's Research Hospital.

RESULTS

In vitro interference by CP1. Interference with standard virus multiplication in vitro was measured by an infectious center reduction assay (10, 20) with Wilson-Smith neurotropic (WSN) virus as the standard virus. The DIU titer of various amounts of CP1 was calculated from cell and infectious center counts by the method of Janda et al. (20) (Fig. 1). For DI preparations of fowl plague virus, such a curve is nonlinear at DIU per cell multiplicities greater than 0.5 to 1 (10), equivalent to 13 μl of CP1 in Fig. 1. From the initial linear region of Fig. 1, we determined that the CP1 virus stock contained 9.8×10^6 DIU/ml. This is comparable to values reported for other DI preparations (20). The same CP1 virus stock contained 7×10^5 PFU/ml. Therefore, a large fraction of CP1 virus particles produce interference with standard viral replication. CP1370 produced negligible interference with WSN virus in this assay.

In vivo interference by CP1. We have confirmed an earlier finding (3) that coinfection of chickens with CP1 protects them from lethal CP1370 infection and additionally have assessed whether protection is due to inhibition of the spread of virulent virus (Table 1). Groups of eight adult hens were doubly infected via the nasal cleft with mixtures of either CP1370 plus CP1 or CP1370 plus A/Duck/Michigan/25/80. Duck/Michigan is an avirulent H5N2 virus without subgenomic RNAs (3). In the Duck/Michigan-CP1370 group, there was 100% mortality within 8 days. In the CP1-CP1370 group, only three of eight birds died within this time; a fourth became sick but recovered; four birds showed no disease

TABLE 1. Interference with pathogenicity of CP1370 by CP1 virus infection in chickens[a]

Expt	Infecting H5N2 viruses	EID_{50}	Disease signs (no. affected/no. inoculated)	Mortality (no. dead/no. inoculated)	Virus detection[b] (no. detected/no. inoculated)	
					Cloaca	Trachea
1[c]	Duck/Michigan/25/1980 + CP1370	$10^7 + 10^4$	6/6	5/6	ND	ND
	CP1 + CP1370	$10^7 + 10^4$	4/14	2/14	ND	ND
2	Duck/Michigan/25/1980 + CP1370	$10^6 + 10^4$	8/8	8/8	7/8	8/8
	CP1 + CP1370	$10^6 + 10^4$	4/8	3/8	3/8	8/8

[a] Groups of adult White Leghorn chickens were infected through the nasal cleft with the indicated amounts of virus. Disease signs and deaths generally occurred between 3 and 6 days after inoculation.

[b] Tracheal and cloacal swabs were obtained from chickens on day 3 after inoculation. Samples (0.1 ml) were injected into embryonated eggs. After incubation for 48 h at 33°C, egg allantoic fluid was tested for HA activity. ND, Not determined.

[c] Data in experiment 1 is from reference 3.

signs (Table 1, experiment 2). We extended this result by collecting tracheal and cloacal swabs from each bird on day 3 after inoculation. Virulent H5N2 viruses reach and traverse the intestinal tract of chickens to appear in feces, whereas avirulent viruses usually do not (unpublished data). On day 3, disease signs were noted for only two birds in the Duck/Michigan-CP1370 group and for none in the CP1-CP1370 group. HA analysis of eggs inoculated with these swabs showed that every bird was harboring virus in the trachea. Cloacal swabs were found positive for HA for 10 of 12 birds that developed disease symptoms during the experiment and for none of the 4 birds that remained disease-free (Table 1). This suggests that the spread of virulent virus was inhibited in protected birds during the first 3 days of infection.

Defectiveness of CP1. To determine if CP1 was defective compared with the virulent CP1370 virus, plaque and hemagglutination titers were determined. The CP1370 virus stock had a plaque-forming/hemagglutinating ratio of 2.8×10^5, which is comparable to infectivity ratios approaching 10^6 for egg-grown fowl plaque virus (9). The CP1 virus stock had a plaque-forming/hemagglutinating ratio of 1.1×10^4, or 25-fold lower than that of the virulent virus. This also is comparable to laboratory-generated fowl plague DI preparations (9). Therefore, CP1 is defective as well as interfering, and it resembles laboratory-induced influenza virus DI preparations in both respects.

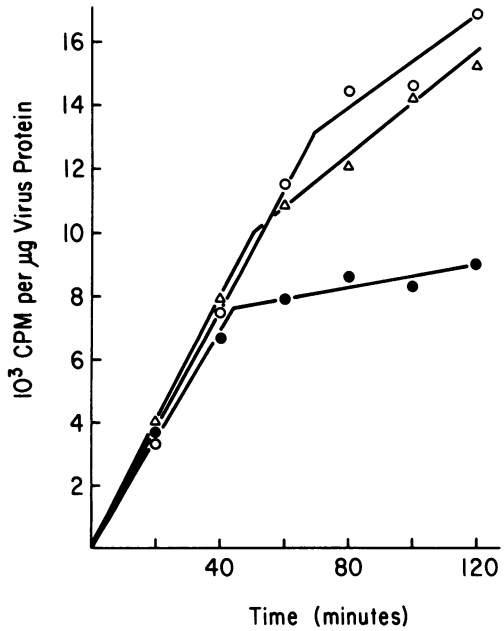

FIG. 2. Kinetics of in vitro transcription by Chicken/Pennsylvania viruses. Reactions were performed as previously described (13, 26), and aliquots were assayed for trichloroacetic acid-insoluble radioactivity. Symbols: ○, CP1 transcription; ●, CP1370 transcription; △ WSN transcription.

Since it has been shown that influenza virus defectiveness is sometimes associated with a reduction in the kinetics of in vitro transcription (1, 4, 9, 13), we next compared the primary transcription activity of CP1 and CP1370 in vitro (Fig. 2). Initial rates of transcription (up to 40 min) were similar for CP1, CP1370, and WSN standard virus. This suggests that CP1 virion RNAs (vRNAs) are not deficient in associated transcriptase enzyme complex, in contrast to results from laboratory-generated DI preparations (1, 13). CP1370 transcription reached a plateau earlier than CP1 or WSN did. This pattern was found in three separate experiments with two different preparations of CP1370. The apparent lability of CP1370 transcriptase may be due to unknown point mutations in the CP1370 polymerase genes.

We also compared the levels of polymerase genes in CP1, CP1370, and WSN virus RNA preparations by densitometric scanning of autoradiographs of Northern blots hybridized with oligodeoxynucleotide probes (Table 2). Results were arbitrarily normalized against nucleoprotein (NP) gene levels in the different RNA tracks. Table 2 shows that CP1 RNA was not deficient in polymerase genes compared with CP1370, again in contrast to the RNAs of laboratory-induced DI preparations (1). Both exhibited less polymerase RNA

TABLE 2. Polymerase gene levels in Chicken/Pennsylvania virus RNA[a]

Virus	Polymerase gene levels with probe:		
	PB1-17	PA-8	PB2-5
CP1370	0.38	0.37	0.13
CP1	0.34	0.32	0.21
WSN	1.0	1.0	1.0

[a] Northern blots of CP1370, CP1, and WSN vRNA were made and hybridized with a molar excess of probes PB1-17, PA-8, PB2-5, or NP-17 (Table 3). Probe binding to polymerase and NP bands was measured by scanning densitometry of autoradiographs, yielding arbitrarily defined counts. Polymerase gene levels are expressed as fractions of the WSN level of the same gene. Results among the various RNA preparations were normalized as follows:

[P (CP)/NP (CP)]/[P (WSN)/NP (WSN)]

where P stands for polymerase gene counts, NP stands for NP gene counts, CP is CP1 or CP1370 vRNA, and WSN is WSN vRNA.

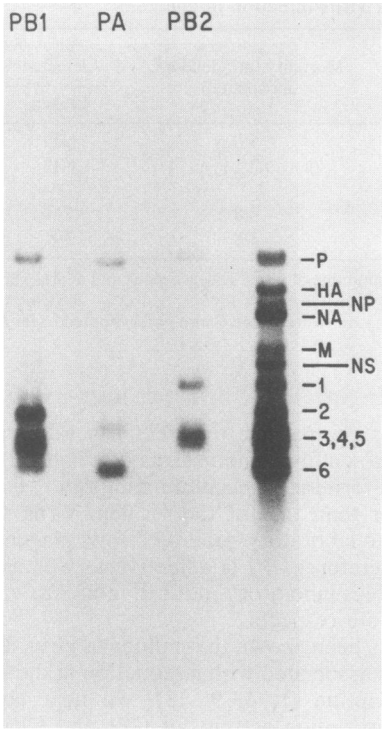

FIG. 3. Northern blot analysis of CP1 RNA. Blots were hybridized with polymerase-specific ^{32}P-labeled probes PB1-17 (lane PB1), PA-8 (lane PA), PB2-5 (lane PB2), or with a probe for the universal influenza A vRNA 5′ terminus (unlabeled lane at right). RNA bands: P, standard polymerase; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural; 1 through 6, subgenomic RNA segments.

than WSN virus did; however, this measurement may reflect unknown differences in the target sequences for the probes.

Primary structure of CP1 subgenomic RNAs. The typical influenza virus DI RNA is derived from one of the polymerase gene segments by internal deletion, so that both ends of the parent gene are conserved (25). Subgenomic RNAs from gene segments other than polymerase have rarely been found (21). Oligonucleotide mapping had indicated that the largest of the CP1 subgenomic RNAs was related to a polymerase gene (3). To confirm that the various CP1 subgenomic RNAs resembled DI RNA in their primary structure, we did Northern blot experiments with 5′-^{32}P-labeled oligodeoxynucleotides as probes. An internal deletion mutant could be identified if the probes for the two ends of a particular gene hybridized to a subgenomic RNA while a probe for the center of the gene did not. This method did not allow us to rule out possible mosaic-rearrangement or multiple-deletion subgenomic structures, but these are rarely observed in influenza virus (21). Figure 3 shows a typical Northern blot, with probes for the specific vRNA 3′ ends of each of the three polymerase genes and for the universal influenza A vRNA 5′ end. Subgenomic RNA segments 1, 2, and 6 (originally identified on polyacrylamide gels) can be resolved on these blots, but several overlapping bands appear in the region labeled 3,4,5.

Table 3 shows the results of the analysis. Specific probes for HA, NP, neuraminidase (NA), matrix (M), and nonstructural (NS) gene segments did not hybridize to any subgenomic RNA, supporting a polymerase gene origin for the subgenomic RNAs. The largest of these (subgenomic RNA 1; estimated length, 750 nucleotides) hybridized only with probes for the two ends of the PB2 gene (PB2-5, PB2-225, and PB2-2284). It did not hybridize with a probe for the center of PB2 (PB2-946). No probe for PB1 or PA hybridized to subgenomic RNA 1. This pattern indicates that subgenomic RNA segment 1 was an internal deletion mutant of PB2. By a similar analysis, subgenomic RNA segment 2 (estimated length, 600 nucleotides) and segment 6 (estimated length, 350 nucleotides) were identified as internal deletion mutants of PB1 and PA, respectively (Table 3). The identity of subgenomic RNAs in the 3, 4, 5 region was unclear, since overlapping bands were not resolved on the autoradiographs. Probes for the two ends of each polymerase gene bound in this region, whereas probes for the centers of the polymerase genes did not bind. Although the possibility of mosaic RNAs cannot be excluded, the most likely interpretation is that different RNAs, each of monogenic polymerase origin, comigrate in this region. Therefore, as internal deletion mutants of polymerase genes, the CP1 subgenomic RNAs are similar in primary structure to the laboratory-induced DI RNAs of influenza virus.

From Fig. 3, it is also apparent that the molar amounts of subgenomic RNA are in excess over the full-length polymerase RNA. Based on densitometry measurements and assuming the PB1, PB2, and PA standard RNAs to be approximately equimolar, the total subgenomic RNA was estimated to be in 18-fold molar excess over total standard polymerase RNA on these blots. This is similar in magnitude to the data of Chanda et al. (13). However, our value may be artificially inflated because of possible differences in transfer efficiency between large and small RNAs. Also, since the CP1 virus stock had been prepared after diluted passages in eggs, these levels of subgenomic RNA may be different from levels present in infected chickens.

Viral subgenomic RNAs in natural influenza virus infections. Although influenza virus DI particles are easily generated in the laboratory by high multiplicity passage, they have not been reported in natural influenza virus infections. The CP1 virus represents only the second report known to us of a natural influenza virus isolate possessing subgenomic RNAs (3, 28). To determine whether subgenomic RNAs occur commonly in natural infections, we examined RNA preparations of a selection of viruses from the influenza virus repository at St. Jude Children's Research Hospital (Table 4). These viruses included isolates from wild and domestic ducks, turkeys, swine, gulls, whales, and seals. A variety of influenza virus subtypes were represented. In most cases, the RNA was prepared from the second egg passage (1:100 dilution) of the original sample. RNAs were examined by polyacrylamide gel electrophoresis followed by silver staining. Of 18 virus preparations examined, subgenomic RNAs were detected in 5 (Table 4). These subgenomic RNAs were usually represented at low levels of mass compared with standard gene levels. To determine if these RNAs were of polymerase gene origin, Northern blots were done on four of the five positive RNA samples by using probes PB1-17, PA-8, and PB2-5. Subgenomic RNAs in each sample hybridized with these probes (Fig. 4). A pair of PB2-derived subgenomic bands (arrows in Fig. 4) appeared nearly identical in the RNA tracks of A/Mallard/Alberta/75/76 and A/Mallard/Alberta/77/76. These were both H3N8 viruses, isolated on the same day from different wild ducks. We were unable to determine if the isolations were made at the same pond. This data suggests transmission of subgenomic RNA-bearing viruses among birds. However, other subgenomic RNAs of these viruses did not comigrate. Two other Mal-

TABLE 3. Hybridization analysis of CP1 subgenomic RNAs

RNA segment	Probe[a]																
	PB2-5	PB2-225	PB2-946	PB2-2284	PB1-17	PB1-1375	PB1-2278	PA-8	PA-1143	PA-1601	PA-2196	HA-44	NP-17	NA-146	M-8	NS-207	Universal 3′ end
PB2	+	+	+	+													+
PB1					+	+	+										+
PA								+	+	+	+						+
HA												+					+
NP													+				+
NA														+			+
M															+		+
NS																+	+
Subgenomic																	
1	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+
2	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+
3, 4, 5	+	−	−	+	+	−	+	+	−	−	+	−	−	−	−	−	+
6	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+

[a] Probes consisted of 5′-^{32}P-labeled oligodeoxynucleotides of 12 to 20 bases which matched the plus-strand sequence of the indicated gene. Numbers are the position on the gene corresponding to the 5′-terminal base of the probe. +, Hybridization of probe to the indicated RNA segment on Northern blots; −, no detectable hybridization of probe to the RNA segment. For standard RNA segments, absence of hybridization is not indicated.

lard/Alberta viruses isolated at nearly the same time (Table 4) did not show subgenomic RNAs detectable by silver staining.

Among other viruses in Table 4 from which subgenomic RNAs were detected, Duck/Minnesota/1086/80 is noteworthy in that extremely low levels of standard polymerase genes were found by both silver staining and Northern blot methods (Fig. 4). We expect this virus preparation to be highly defective. Reduction in polymerase RNA content has also been observed in several influenza virus DI preparations (1, 25). Guinea fowl/New York/13801/86 was an isolate from a recent H5N2 poultry epidemic in the eastern United States, the agents of which partly resemble CP1 (W. Bean, personal communication). Its RNA was not analyzed on Northern blots. Together, the above results suggest that subgenomic RNAs are sometimes produced and packaged into virions in natural influenza virus infections. It is not known if the viruses in Table 4 that contain subgenomic RNAs have interfering activity.

TABLE 4. Detection of subgenomic RNAs in influenza virus isolates from natural infections[a]

Virus	Subgenomic RNAs
Mallard/Alberta/16/76 (H3N8)	−
Mallard/Alberta/75/76 (H3N8)	+
Mallard/Alberta/77/76 (H3N8)	+
Mallard/Alberta/92/76 (H4N6)	−
Mallard/Alberta/586/84 (H6N8)	−
Mallard/Pennsylvania/10218/84 (H5N2)	−
Domestic duck/Minnesota/1086/80 (H4N8)	+
Gull/Maryland/704/77 (H13N6)	+
Gull/Massachusetts/26/80 (H13N6)	−
Ruddy Turnstone/New Jersey/65/85 (H7N3)	−
Turkey/Minnesota/522/78 (H6N1)	−
Turkey/Minnesota/833/79 (H4N2)	−
Turkey/Kansas/4880/80 (H1N1)	−
Guinea Fowl/New York/13801/86 (H5N2)	+
Swine/Tennessee/1/75 (H1N1)	−
Swine/Italy/526/85 (H3N2)	−
Whale/Maine/328HN/84 (H13N9)	−
Seal/Massachusetts/1/80 (H7N7)	−

[a] Viruses were grown in eggs and RNA prepared as previously described (5). RNAs were examined by electrophoresis on 3% polyacrylamide–7 M urea gels followed by silver staining for presence (+) or absence (−) of subgenomic RNAs.

DISCUSSION

Our data indicate that CP1 virus contains DI particles. CP1 had a low plaque-forming/hemagglutinating ratio, and it interfered with infectious center formation by standard WSN virus in vitro. The subgenomic RNAs detected in CP1 preparations (3) are internally deleted polymerase RNAs, like the DI RNAs of laboratory-generated DI influenza virus populations. CP1 thus appears to be a DI influenza virus strain which was produced and transmitted in natural influenza virus infections.

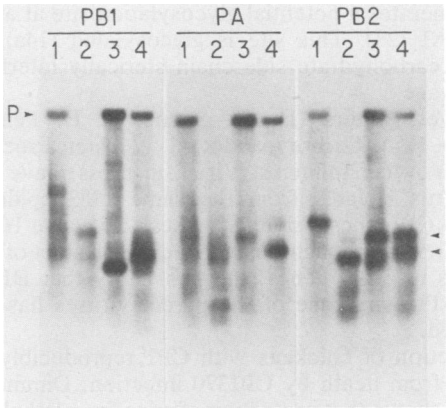

FIG. 4. Northern blot analysis of subgenomic RNAs from natural influenza virus isolates. Blots were hybridized with polymerase-specific probes PB1-17 (lanes PB1), PA-8 (lanes PA), or PB2-5 (lanes PB2). RNAs were from the following virus preparations: Gull/Maryland/704/77 (lanes 1); Domestic duck/Minnesota/1086/80 (lanes 2); Mallard/Alberta/77/76 (lanes 3); Mallard/Alberta/75/76 (lanes 4). P indicates the positions of standard polymerase genes. Arrows at right indicate comigrating subgenomic RNAs from different virus isolates (see text).

Subgenomic RNAs were also detected in RNA preparations of 5 of 18 influenza virus isolates from animals (Table 4). Thus, subgenomic RNAs characteristic of DI particles are sometimes generated and amplified in natural influenza virus infections, not just under laboratory conditions. We have no data, pro or con, that these five viruses with subgenomic RNAs produce interference. Nayak et al. (25) believe that the low virus per cell multiplicity of a natural infection is not favorable for the survival of defective viruses which require high multiplicity for rescue by complementation. However, influenza virus replicates to very high titers in the intestinal tracts of ducks; titers up to 6×10^7 EID_{50}/ml in intestinal mucosa and 6×10^8 EID_{50}/ml in feces have been reported (32). This may be sufficient for the amplification of DI particles in ducks.

We have considered the possibility that DI particles arose during egg passages made after the CP1 virus sample was isolated from chickens. However, these passages were done at low multiplicities of infection and included two limit-dilution passages. These conditions do not favor amplification of DI particles (25). On polyacrylamide gels, CP1 RNA prepared in the first egg passage of the original virus isolate was similar to RNA made from the last egg passage (unpublished data). This suggests that DI particles were already present in the original isolate.

CP1 was not defective compared with CP1370 in either the kinetics of in vitro transcription or the levels of polymerase genes. This is in contrast to the results with laboratory-generated DI preparations of Akkina et al. (1) and Chanda et al. (13). Conceivably, passage in animals or eggs selected for primary transcription-competent virus populations. We note also that Akkina et al. (1) and Chanda et al. (13) were able to directly compare their DI preparations with the parent standard virus preparation. CP1370 is not the standard parent of CP1 (24). Therefore, our results on transcription kinetics and polymerase gene levels do not necessarily contradict the conclusion that CP1 contains DI particles.

Other factors beside DI particles contribute to the lack of virulence of CP1. Cleavability of the HA glycoprotein into HA1 and HA2 has been correlated with avian influenza virus pathogenicity for chickens (8). Sequencing of the CP1 HA gene has revealed a point mutation (relative to CP1370 HA) which generates a potential glycosylation site at amino acid 11 of HA1 (23). This site is glycosylated (14a), and the resulting carbohydrate side chain sterically interferes with HA cleavage.

However, others (D. A. Senne, J. E. Pearson, Y. Kawaoka, E. A. Carbrey, and R. G. Webster, Proc. 2nd Intl. Symp. on Avian Influenza Virus, in press) have identified H5N2 virus isolates from the same 1983 epidemic that produced CP1; these isolates possess cleavable HA but are still avirulent. Although this may be the product of unknown mutations in other genes, the possibility that DI particles suppress the virulence of the parent viruses has not been discounted.

Coinfection of chickens with CP1 reproducibly protects chickens from death by CP1370 infection. Dimmock et al. (16) have shown that protection of mice from lethal influenza virus by DI particles is not due to inhibition of virus multiplication in lung extracts, induction of interferon, increase in antigen levels, or increase in neutralizing-antibody response. They speculate that, aside from the conventional DI-mediated inhibition of standard virus replication, a second protective activity operates in the mouse system by modulation of host T-cell regulatory networks. Rabinowitz and Huprikar (27) also argue that interference in vivo is related to augmentation of the host immune response. For protection of chickens by CP1, modification of host immune response appears to be not involved, since a serologically related avirulent H5N2 virus, Duck/Michigan/25/80, had no protective effect.

Alternatively, in considering DI-mediated protection of mice from encephalitis by vesicular stomatitis virus, Cave et al. (11, 12) argue that protection is indeed a function of inhibition of virus multiplication. However, in their model this function takes a cyclic form over several generations of virus replication, and the relative DI levels for protection in vivo cannot easily be predicted based on the DI levels for inhibition of cytopathic effect in vitro. Low DI levels may sometimes produce a significant degree of protection (11). Therefore, it remains conceivable that DI particles associated with CP1 suppress the multiplication of the virulent virus in chickens. The detection of virus 3 days after inoculation from cloacal swabs of unprotected, but not from protected, chickens supports this hypothesis. Studies are underway, by careful examination of infected chicken organs for virus, to determine whether protection against virulent virus by CP1 in the chicken model is due to inhibition of virulent virus multiplication.

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