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cDNA Cloning of the Messenger RNAs of Five Genes of Canine Distemper Virus

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SUMMARY

Messenger RNAs from Vero cells infected with the Onderstepoort strain of canine distemper virus (CDV) were cloned into the PstI site of plasmid pAT153. Total polyadenylated RNA was used and resulting clones were screened with 32P-labelled cDNA probes from infected and mock-infected cells. The virus specificity of the clones was proven by Northern blot hybridization and by ability to select radioactive virus mRNAs labelled in vivo in the presence of actinomycin D. Clones from the N, P and M genes of CDV were identified by hybrid select translation; clones which presumably represent the H and F genes were also obtained. The clones allowed a designation of the major viral mRNA bands. Bicistronic mRNAs were identified, and their selection by various clones suggests a gene order of 3'-N-P-M-70K-65K-L-5' for this virus.

INTRODUCTION

The study of canine distemper virus (CDV) and the closely related measles virus (MV) has been of interest during the past 15 years because of the involvement of these viruses in long-term neurological diseases in their natural hosts (ter Meulen & Carter, 1982). Subacute sclerosing panencephalitis in man and old dog encephalitis in dogs are associated with persistent infections by MV and CDV respectively. The mechanisms by which persistent infections are established and maintained are as yet not well understood in molecular terms and, therefore, gene-specific probes of MV and CDV are required to study persistent infections at the level of genome replication and transcription. Gene-specific probes have recently been produced for four genes of MV (Gorecki & Rozenblatt, 1980; Rozenblatt et al., 1982; Billeter et al., 1984; Bellini et al., 1984) and a clone specific for the nucleocapsid (N) gene of CDV has been described (Barrett & Mahy, 1984).

Here we report the isolation of cDNA clones for five genes of CDV, in addition to a characterization of the major mRNA species found in infected cells. Poly(A)-containing RNAs (pA+RNA) have been analysed by gel electrophoresis in MV-infected cells (Hall et al., 1978; Rozenblatt et al., 1982; Udem & Cook, 1984) and in CDV-infected cells (Barrett & Mahy, 1984). By using our cDNA clones for selection of radioactive mRNAs and hybrid select translation we were able to designate the coding specificity of most of the pA+RNA species found in the infected cells.

METHODS

Cells and viruses. Vero cells (African green monkey kidney cells) were grown in Eagle's medium supplemented with 8% (v/v) newborn calf serum and antibiotics (Campbell et al., 1980). A large plaque variant of the Onderstepoort strain of CDV (Cosby et al., 1981) was grown and passaged as described earlier (Campbell et al., 1980). Virus stocks and Vero cells were routinely tested for the absence of contaminating mycoplasma, as described by Simpson et al. (1983).

Extraction of polyadenylated RNA as cloning template. RNA was extracted from Vero cells infected with CDV at an m.o.i. of 0.1 by the method described by Glisin et al. (1974) and Martin & ter Meulen (1976). At 22 h post-infection actinomycin D (Sigma) was added to a final concentration of 2 μg/ml and at 24 h, when c.p.e. had
engulfed most cells, the cellular monolayers were solubilized in 4% (w/v) sodium laurylsarcosinate in 0.1 M-Tris-HCl pH 8. After reduction of the viscosity of the sample by force-pipetting through a narrow-gauge syringe needle, ultrapure CsCl (Bethesda Research Laboratories) was added to a concentration of 1 g/ml and then layered over a 57 m- CsCl, 0.1 M-EDTA pH 6.5 cushion and centrifuged for 16 h at 35000 g. The RNA pellet was resuspended in 0.5 M-NaCl, 0.1% sarcosinate, 1 mM-EDTA, 10 mM-Tris-HCl pH 7.5, denatured by heating at 65°C for 3 min and cooled quickly on ice. The RNA was enriched for pA+ species by oligo(dT) affinity chromatography as described by Aviv & Leder (1972).

Isotopic labelling of cells. The analysis and labelling of CDV proteins has been described previously (Campbell et al., 1980).

Actinomycin-treated cells were labelled with [3H]uridine for 2 h during the late stages of the infection, and the RNA extracted and purified as described above. Labelling with [3P]orthophosphate (Amersham) was carried out in phosphate-free medium (Gibco) containing 2% dialysed newborn calf serum and 2 μg/ml actinomycin D supplemented with 32PO4- at 200 μCi/ml. Cells were pretreated with phosphate-free medium 1 h prior to the addition of the radioactive label.

Cloning procedure. pA+RNA from infected cells was reverse-transcribed in 50 mM-Tris-HCl pH 8.2, 10 mM-magnesium acetate, 10 mM-dithiothreitol, 40 mM-NaCl, 1 mM each of dATP, dTTP and dGTP, 0-4 mM-[3H]dCTP (sp. act. 1 mCi/lμmol), 100 μg/ml actinomycin D, 20 μg/ml oligo(dT)2-18, 20 μg/ml pA+RNA and 400 units/ml reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.) by incubation at 37°C for 2 h. The reaction was terminated by addition of an equal volume of 100 mM-EDTA and extracted with phenol–chloroform (90:10) saturated with TE buffer (10 mM-Tris–HCl pH 7.4, 1 mM-EDTA) and passed over a Sephadex G-50 column. The nucleic acid present in the void volume was precipitated with 2 vol. ethanol. About 700 ng of the cDNA/mRNA hybrid was directly extended with approximately 10 dCMP residues using the homopolymer procedure described by Roychoudhury et al. (1976) using terminal transferase (P-L Biochemicals), phenol-extracted and passed over a Sephadex G-50 column. PstI-restricted pAT153 (5 μg) was extended with an average of seven dGMP residues by the same technique. For transformation experiments 50 ng of tailed plasmid and 5 ng of tailed cDNA/mRNA hybrid were co-precipitated with ethanol and the pellet was resuspended in 20 μl 100 mM-NaCl, 10 mM-Tris–HCl pH 7.6, 1 mM-EDTA, heated to 68°C for 30 min and allowed to cool slowly to room temperature overnight. The entire mixture was used to transform Escherichia coli cells HB101 or ED8767 prepared according to the method of Dager & Ehrlich (1979). Tetracycline-resistant ampicillin-sensitive colonies were selected. Colony hybridizations were performed according to the method of Grunstein & Hogness (1975) using 32P-labelled cDNA probes (2 × 108 c.p.m./μg cDNA) prepared by reverse transcription of pA+RNA from infected and mock-infected cells as described above in the presence of 32P-labelled dCTP.

Selection of RNA by recombinant plasmid DNA. Plasmid DNA was extracted from various clones by the method of Marko et al. (1982). Twenty to 50 μg of recombinant plasmid DNA was linearized with EcoRI and bound to nitrocellulose discs (1 cm diam.) after denaturation. The discs were soaked in 0.5 mM-NaOH, 1 mM-NaCl for 10 min, blotted dry, and soaked in 2 mM-Tris–HCl pH 7.0 for 10 min, blotted dry and soaked in 2 × SSC (SSC is 0.15 M-NaCl, 0.015 M-sodium citrate). The DNA was bound to the filters by baking at 80°C for 2 h. The filters were cut up into filter fragments and washed five times with 2 × SSC, 0.1% SDS at room temperature and twice at 50°C with 0.1% SSC, 0.1% SDS. The RNA was released by heating the filters twice in 150 μl sterile H2O at 100°C for 2 min. The RNA was coprecipitated with 10 μg glycogen by addition of 2 vol. ethanol. The RNA was then either used to direct protein synthesis in rabbit reticulocyte lysates (Amersham) in the presence of 25 μCi [35S]methionine or analysed on denaturing gels.

Gel analysis of RNA and Northern blotting. Radiolabelled RNAs were fractionated on 1-5% agarose gels containing 2 M-formaldehyde as described by Barrett & Mahy (1984). Following electrophoresis gels were either dried directly on to Whatman 3MM paper for autoradiography or after impregnation with 10% diphenyloxazole in ethanol-dioxan mixtures (80:20) for fluorography. Unlabelled RNAs were fractionated on gels and blotted on to nitrocellulose as described by Barrett & Mahy (1984) and were prehybridized and hybridized with nick-translated plasmid DNA as described by Thomas (1980). Nick translation was carried out in the presence of [32P]dCTP by the method of Rigby et al. (1977) to provide plasmid DNA probes with specific activities ranging from 8 × 108 to 2 × 1010 c.p.m./μg.

In some experiments, RNAs were fractionated on 7.5% methylmercuric hydroxide (Lancaster Synthesis Ltd) 1-5% agarose gels made up in borate buffer [500 mM-boric acid, 50 mM-sodium tetraborate, 100 mM-sodium sulphate, 10 mM-EDTA, 5% (w/v) SDS, pH 8.19]. All samples were taken up in 30 μl of sample buffer consisting of 3 μl glycerol/bromophenol blue, 15 μl borate buffer, 1.5 μl 1 M-methylmercuric hydroxide, 10-5 μl H2O.
RESULTS

RNA species in infected cells

Virus-induced RNA species were labelled with \([^{32}\text{P}]\)orthophosphate or \([^{3}\text{H}]\)uridine in the presence of actinomycin D when c.p.e. had engulfed most of the cells in large syncytia. Fig. 1 shows the profiles obtained from RNA samples analysed on denaturing gels containing formaldehyde or methylmercuric hydroxide. In total, ten virus-induced bands could be identified with mol. wt. ranging from 0.46 x 10^6 to 1.8 x 10^8. The most abundant species were in band number 2/3 which in some gels and from later designate experiments appeared to contain two RNAs.

A comparison of RNA from cells infected with measles virus and mumps virus (data not shown) indicated that there were some host cell-derived bands common to all three viruses which were synthesized in the presence of actinomycin D. The origin and specificity of these bands are currently under investigation. The numbered bands were later shown to be virus-specific and their coding specificity is described below.

After separation of RNAs into pA⁺ and pA⁻ fractions it was clear that the pA⁻ fraction contained labelled RNA species which were smaller than the mRNA species. We could not detect the presence of genomic or defective interfering (DI) particle RNA in the pA⁻ fractions.

Fig. 1. Analysis of RNA labelled in vivo on denaturing gels. (a) \([^{3}\text{H}]\)Uridine-labelled RNAs analysed on a methylmercuric hydroxide-agarose gel. Lane 1, Vero ribosomal RNAs; lane 2, pA⁺ RNA from CDV-infected Vero cells labelled in the presence of actinomycin D. (b) \([^{32}\text{P}]\)-labelled RNAs analysed on a formaldehyde-agarose gel. Lane 1, total infected cell RNA labelled in the presence of actinomycin D; lane 2, pA⁻ RNA; lane 3, pA⁺ RNA.
Translation of pA+ RNAs in vitro

The starting material for cDNA cloning experiments was characterized by its ability to direct the synthesis of virus-specific proteins in rabbit reticulocyte lysates. RNA was extracted at different times post-infection from CDV-infected cells and a time course study (data not shown) indicated that RNAs extracted between 14 and 23 h were equally able to direct the synthesis of virus-specific proteins in an \textit{in vitro} system. RNA was routinely extracted at 19 h and fractionated into pA+ and pA− fractions. The pA+ fraction directed the synthesis predominantly of the N, P, M and S proteins (Fig. 2a), which have been described previously as virus-induced protein in infected cells (Campbell \textit{et al.}, 1980; Rima, 1983). The identity of the proteins synthesized \textit{in vitro} was confirmed by limited proteolysis of the proteins synthesized \textit{in vivo} and \textit{in vitro} with \textit{Staphylococcus aureus} V8 protease. Fig. 2(b) confirms the identity of the N, P, M and S proteins. Precursor proteins for the H and F proteins of CDV have not been identified in these experiments.

These RNA preparations were considered to be suitable starting material for the cloning experiments.

\textit{Preparation of cDNA clones}

Poly(A)+ RNA from CDV-infected Vero cells was reverse-transcribed after priming with oligo(dT) and the resulting cDNA/mRNA hybrids were tailed directly with dCMP residues
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Fig. 3. Selection of radioactive mRNAs by nitrocellulose-bound plasmid DNA. $^{32}$P-labelled RNAs were hybridized to filter-bound DNA, eluted and analysed on formaldehyde-agarose gels. (a) Selection from $^{32}$P-labelled pA-trRNA from infected cells by various pCDV clones. (b) Selection from total $^{32}$P-labelled RNA from infected cells by various pCDV clones and a pAT153 control.

(Van der Werf et al., 1981) and cloned after annealing with equimolar amounts of dGMP-tailed, PstI-restricted pAT153 DNA. The recombinant plasmids were transferred into E. coli. In one experiment we obtained from 7 ng of tailed hybrid 30 cDNA clones of the correct antibiotic phenotype. In a second experiment we obtained 108 cDNA clones from 25 ng of hybrid. The cDNA clones were analysed by colony hybridization carried out by the Grunstein & Hogness procedure (1975) with $^{32}$P-labelled cDNA probes from CDV-infected and mock-infected Vero cells. Duplicate filters were hybridized with the plus (CDV-infected cell cDNA) and minus (mock-infected cell cDNA) probes. 34 clones which gave the correct response in this plus/minus probing were considered to be possible virus-specific clones and were analysed further. They represent 38% and 20% of the total number of clones with the correct phenotype in the two experiments.

**Identification of clones by selection of radioactive RNA**

Plasmid DNA from clones that hybridized only with the CDV-infected cell cDNA probes were purified by CsCl centrifugation and the DNAs were restricted with PstI to release the inserts. The sizes of these were estimated on polyacrylamide gels. The clones containing plasmid with the longest inserts were used in further analysis.

In order to determine which of the mRNAs had been cloned, plasmid DNA, bound to nitrocellulose, was hybridized with radioactive RNA labelled in vivo in the presence of actinomycin D. The RNAs selected by various plasmid DNA clones were analysed on formaldehyde–MOPS gels. All the virus-specific cDNA clones were analysed in order to group them into classes. The results for some of the largest clones are shown in Fig. 3.

This analysis showed that clones 6, 7, 16, 22, 28, 30 and 41 selected RNA that co-migrated with doublet pA+ RNA band 2/3; clones 1 and 51 selected RNA band 4. When these gels were exposed for longer periods it became apparent that some of the clones also selected some of the RNA bands (6 to 9) and these, therefore, probably represented bicistronic mRNAs similar to those observed in Newcastle disease virus (NDV) and respiratory syncytial virus (RSV)-infected cells (Collins et al., 1982; Collins & Wertz, 1983).
Fig. 4. Hybrid select translation. RNAs selected by various cloned cDNAs (numbers indicated above various lanes) were used to direct in vitro protein synthesis. The products were analysed by SDS–PAGE on 8 to 15% gradient gels (b, c) or 10% gels (a, d). L, Lysate band; i.v.m., infected cells labelled with [35S]methionine in vitro; i.v.t., in vitro translation of total RNA.
Table 1. Characteristics of CDV clones

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<tr>
<th>Clone</th>
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<th>Designation</th>
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<tr>
<td>1</td>
<td>1060</td>
<td>M</td>
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<tr>
<td>2</td>
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<td>70K protein</td>
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<tr>
<td>3</td>
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<td>51</td>
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<td>54</td>
<td>650</td>
<td>65K protein</td>
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On the basis of selection of bicistronic mRNAs, the clones that selected RNAs co-migrating with the double band 2/3 were separated into two groups, i.e. those which selected only one bicistronic mRNA (band 7) such as clones 6, 7, 16 and 28, and those which selected two bicistronic mRNAs (such as 30, 41 and 22) which selected bands 6 and 7. Clone 54 selected only one of the slowest migrating bicistronics (band 9) and clone 2 also selected bands 8 and 9 (data not shown). It was also noted that some of the clones selected some of the other minor mRNAs weakly. This probably does not represent non-specific binding as in control selection with the cloning vector pAT153 no labelled RNA was selected at all. The possibility that the cloned DNAs consist of sequences from DI particle genomes or bicistronic mRNAs cannot be ruled out at present. However, this explanation is less likely, since the amount of this hybridization varied from experiment to experiment.

Northern blot analysis and hybrid select translation

The virus-specific nature of many of the clones was confirmed by hybridization of nick-translated plasmid DNAs to Northern blots of CDV-infected cells and mock-infected cell RNAs. Poly(A)^+ RNA was separated on denaturing agarose-formaldehyde gels and blotted on to nitrocellulose. ^32P-labelled plasmid DNA of the viral clones labelled by nick translation did not hybridize with mock-infected cell RNA and hybridized strongly with pA^+RNA from infected cells (data not shown). Such experiments confirmed the virus specificity of clones pCDV30, pCDV28, pCDV1 and pCDV2.

In order to determine the specificity of the five classes of clones that selected mRNA bands 1, 2 or 3, 4 and 5 we attempted to carry out translation experiments in vitro of mRNAs selected by plasmid DNA of representative clones of each of the classes as described in Methods. When RNA selected by plasmid vector pAT153 DNA was added to the in vitro protein synthesis system no other bands were found other than those present in lysates to which no RNA had been added (Fig. 4). However, RNA selected by clone 28 directed the synthesis of a protein that co-migrated with the N protein synthesized in vivo or in vitro. Clones 1, 3 and 51 selected mRNA which directed the synthesis of the P protein of CDV in addition to smaller proteins with mol. wt. 42K, 23K, 16K and a small amount of the N protein. The 23K protein co-migrated with the S protein earlier identified in translation products of the virus in vivo. The N protein synthesized by RNAs selected by these clones could either result from non-specific hybridization or from the selection and translation of bicistronic mRNAs. We favour the second explanation since the M clone (pCDV51) selects RNAs that give rise to a small amount of P and particularly S protein, which may be derived from the translation of P-M bicistronic mRNA.

The RNA selected by clone 2 directed the synthesis of a protein of about 70K. Hybrid select translation experiments with clone 54 suggested that it codes for a 65K protein. The designation of these as H and F precursors is currently under investigation. The designation of the clones and insert sizes are given in Table 1.
This paper describes the synthesis and identification of cDNA clones specific for five genes of CDV. The identification of N, P and M gene-specific clones could be made unambiguously because translation of hybrid-selected RNAs by these clones \textit{in vitro} enabled us to correlate mRNA bands, clones and proteins. The results indicate that the mRNAs for the P and N proteins of CDV are of approximately equal size (i.e. 1700 nucleotides in length) which is comparable to the size and co-migration pattern of measles virus P and N mRNAs (Bellini et al., 1984; Rozenblatt \textit{et al.}, 1982). Our results are in agreement with those obtained for the N gene of CDV by Barrett & Mahy (1984). Translation of RNA selected by a number of CDV clones allowed us to identify unambiguously the smallest poly(A)$^+$ RNA as coding for the M protein. The length of this RNA was about 1450 nucleotides which is similar to that found for the M mRNA of measles virus (Rozenblatt \textit{et al.}, 1982).

It is of interest to note that clones specific for the P gene seem to select RNAs that directed the synthesis \textit{in vitro} not only of the P protein but also of a number of smaller polypeptides, one of which co-migrates with the S protein identified \textit{in vivo} and \textit{in vitro}. The other proteins have approximate mol. wt. of 42K and 16K. These proteins have not yet been identified \textit{in vivo} in labelled CDV-infected cell lysates but they are present in rabbit reticulocyte lysates stimulated with pA$^+$ RNA from CDV-infected cells. The identity of the CDV 42K protein is unknown but in MV-infected cells we have previously identified a protein designated X (Rima \textit{et al.}, 1981) which was found to have extensive peptide homology with the P protein. This X protein is made \textit{in vivo} and \textit{in vitro} (Carter \textit{et al.}, 1983) and it is interesting to note that one of the early measles clones obtained by Rozenblatt \textit{et al.} (1982) and now shown to be a P clone by Bellini \textit{et al.} (1984), also directs the synthesis of a 40K protein.

Two further clones pCDV2 and pCDV54 have been isolated which select mRNA bands 5 (2200 nucleotides) and 4 (1950 nucleotides) respectively and in hybrid select translation direct the synthesis of a 70K and a 65K protein respectively. It is at present difficult to assign definitive coding specificity to these mRNAs although they presumably code for the H and F proteins. In MV-infected cells the largest mRNA (2180 nucleotides) was shown to code for a 65K protein (Rozenblatt \textit{et al.}, 1982), but although this was suggested to be an unglycosylated F protein precursor this designation is now uncertain. Experiments on the designation of pCDV2 and pCDV54 are currently being carried out. We have not identified an mRNA or clone for the L gene although a candidate mRNA (band 10) with mol. wt. $2 \times 10^6$ was prevalent in all RNA profiles.

The four bands (6 to 9) of pA$^+$ RNA labelled \textit{in vivo} were selected by various cDNA clones and this indicates that these are bicistronic mRNAs. Such RNAs have been suggested to be present by Barrett & Mahy (1984) and we suggest that in order of increasing size they represent the P–M, N–P, M–70K and 70–65K bicistronic transcripts. Bicistronic transcripts have also been shown to be present in MV-infected cells (Udem & Cook, 1984), mumps virus-infected cells (J. A. Curran \textit{et al.}, unpublished observations) and NDV- and RSV-infected cells (Collins \textit{et al.}, 1982; Collins & Wertz, 1983).

Assuming that in CDV there is a single promoter, and taking account of the intensity of mRNA bands as well as the number of clones and pattern of bicistronic mRNAs we would suggest the following gene order for CDV.

\textbf{Genes $3'-N-P-M-(70K-65K)-L-5'$; mRNAs $3'-2-3-1-5-4-10-5'$}

Although this corresponds well with that described for other paramyxoviruses such as Sendai virus (Blumberg \textit{et al.}, 1984) an unambiguous decision regarding the order of the H and F genes in CDV cannot be made until the glycoprotein precursors are fully characterized.

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