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Genotypic Variation among Six Isolates of Lactate Dehydrogenase-elevating Virus

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SUMMARY

In the present study, six lactate dehydrogenase-elevating virus (LDV) isolates obtained independently from inbred mice were compared by RNA oligonucleotide fingerprint analysis. The genome RNAs of four of the isolates gave unique fingerprint patterns. The patterns obtained for the other two isolates were similar, but not identical to one of the four unique patterns. These results indicate that more than one genotype of LDV exists and that virus isolates can be grouped by genotype. We have also demonstrated the presence of a 3'-terminal poly(A) tract by direct sequencing of 3'-32P-labelled LDV genome RNA. The presence of a 3'-terminal poly(A) tract distinguishes LDV from the members of the family Flaviviridae, which lack a 3'-poly(A), and justifies the current classification of LDV within the family Togaviridae.

INTRODUCTION

Lactate dehydrogenase-elevating virus (LDV) virions are spherical with an average diameter of 50 to 55 nm. Virions are composed of three structural proteins (Brinton-Darnell & Plagemann, 1975; Michaelides & Schlesinger, 1973): Vp3 (24000 to 44000 mol. wt.), a glycosylated envelope protein; Vp2 (18000), a non-glycosylated, envelope-associated protein; and a capsid protein, Vp1 (15000). The genome is a single-stranded RNA of positive polarity and approximately 14 kb in size (Darnell & Plagemann, 1972). Nothing is currently known about the structure of the viral genome nor about the non-structural proteins encoded by it.

LDV infection leads to a lifelong viraemia in all strains of inbred mice (Mus musculus), but this virus does not infect other host species (Riley, 1974). In infected mice, LDV persists as circulating infectious virus–antibody complexes (Notkins et al., 1966, 1968), but infected mice do not develop immune complex-related diseases (Porter & Porter, 1971). Because of the infectivity of LDV-immune complexes, significant neutralization by murine antiviral antibody can not be demonstrated (Cafruny & Plagemann, 1982). Also, antiviral antibody has proved difficult to produce in other animal species (Cafruny & Plagemann, 1982). The target cell for LDV replication is an uncharacterized, minor subpopulation of primary mouse macrophages and monocytes. Virus–antibody complexes can apparently infect susceptible macrophages via cell Fc receptors (Cafruny & Plagemann, 1982). There appear to be additional specific intracellular factors required for LDV replication, since LDV cannot infect transformed murine macrophage cell lines nor primary macrophages from any other host species (Brinton, 1982).

LDV causes no obvious cytopathic effect in primary mouse macrophage cultures and, therefore, virus infectivity cannot be titrated by plaque assay. However, a number of serum enzymes are elevated in LDV-infected mice. The level of lactate dehydrogenase (LDH) is most affected, increasing about 10-fold by 4 days after infection (Notkins, 1965). This increase in plasma enzyme levels is primarily due to a virus-induced decrease in the rate of clearance of certain enzymes which are normally turned over (Riley et al., 1965; Brinton & Plagemann, 1983). The characteristic plasma LDH elevation is used as the endpoint for virus titration.
The demonstration that several inbred mouse strains are susceptible to LDV-induced central nervous system diseases and that one isolate, designated LDV-C, is more pathogenic than other LDV isolates (Martinez et al., 1980; Stroop & Brinton, 1983) indicated that LDV isolates could differ in their biological characteristics. Because of technical difficulties inherent to LDV infections, characterization of individual LDV isolates by classical methods of serology has not been possible. We report here the use of oligonucleotide fingerprinting as one means for comparison of LDV isolates.

METHODS

**Viruses and animals.** The six isolates of LDV used in the present study were designated LDV-1 to LDV-5 and LDV-C. LDV-1 was isolated by M. Brinton and P. G. W. Plagemann from a C3H mouse which had been injected with tumour 43 cells and was subsequently passaged eight times in Swiss mice (Brinton-Darnell & Plagemann, 1975). LDV-2 was isolated by A. L. Notkins from a mouse that had been injected with a stock of Moloney leukaemia virus. Subsequent passages of this isolate were carried out in CAF-1 mice (Notkins et al., 1963). LDV-3 was isolated by S. Schlesinger from a BALB/c AmN mouse, which had been injected with plasmacytoma MOPC-315 cells and was subsequently passaged in BALB/c AmN, HA/ICR and Swiss/ICR mice (Michaelides & Schlesinger, 1973). LDV-4 was isolated by V. Riley from a Swiss/ICR mouse which had been injected with Ehrlich ascites cells and was subsequently passaged in Swiss/ICR and crf-1 mice (Riley & Wroblewski, 1960; Riley et al., 1960). We obtained LDV-4 from the American Type Culture Collection in 1978 (Martinez et al., 1980). In a subsequent investigation of the history of the LDV-4 virus, we found that an LDV stock was sent to W. Rowe by V. Riley in 1966 and subjected to about 10 mouse passages before it was supplied to the American Type Culture Collection in 1978 by M. Collins, then of Microbiological Associates, Rockville, Md., U.S.A. LDV-5 was obtained directly from M. Collins in 1978 and was an earlier passage of LDV-4. LDV-C was isolated by D. Martinez and M. Brinton from C58 mice which had been injected with Ib leukaemia cells obtained from W. Murphy (Martinez et al., 1980) and was subsequently passaged twice in Swiss mice (Charles River Breeding Laboratories, Portage, Mich., U.S.A.).

LDV-C is phenotypically distinct from the other LDV isolates in that it is very efficient at inducing poliomyelitis in a few genetically susceptible inbred strains of mice (Martinez et al., 1980; Stroop & Brinton, 1983).

Stock plasma pools of the six LDV isolates were prepared by injecting twenty 1- to 2-month-old random-bred Swiss mice (Charles River) intraperitoneally with $10^7$ ID$_{50}$ of virus and then obtaining blood at 18 and 24 h after infection, from the retroorbital sinus with heparinized capillary pipettes. Plasma was separated by low-speed centrifugation, diluted 10-fold with phosphate-buffered saline (PBS), clarified, and samples were stored frozen at $-70^\circ$C. Infectivity titres of these pools determined in mice (Notkins, 1965) ranged from $10^{10}$ to $10^{11}$ ID$_{50}$/ml.

One- to 2-month old C58/J mice (Jackson Laboratories, Bar Harbor, Me., U.S.A.) were used for all further experiments, since we felt that the use of a single inbred mouse strain would yield more consistent experimental results. C58 mice are of special interest because they are susceptible to LDV-C-induced poliomyelitis (Martinez et al., 1980).

**Growth, radiolabelling, and purification of virus.** LDV was grown in either primary mouse peritoneal macrophage cultures or in mice (Brinton-Darnell et al., 1975; Brinton, 1982). The preparation of peritoneal macrophage cultures has been described previously (Brinton-Darnell et al., 1975). Peritoneal macrophages in five T75 flasks were infected with LDV after 1 day in culture at a m.o.i. of 100. After a 1 h adsorption period at room temperature, phosphate-free MEM containing 5% dialysed foetal calf serum was added. Two h later, the cultures were supplemented with 1 mCi of $^{32}$P. Culture fluid was harvested 26 h after infection and clarified by low-speed centrifugation. Virus was pelleted from the culture fluid at 24500 r.p.m. for 2 h at 4°C in a SW28 rotor and resuspended in PBS at 4°C. Virions were first sedimented on a 10 to 38.5% glycerol–PBS gradient in a SW28 rotor at 22000 r.p.m. for 150 min at 4°C. The virion peak was located by obtaining Cerenkov counts of the fractions. The pooled virus-containing fractions were diluted with an equal volume of medium and layered onto a 20 to 75% glycerol gradient which was centrifuged in a SW28 rotor at 24500 r.p.m. for 16 h at 4°C. Peak fractions were pooled and the virions precipitated with ethanol at $-70^\circ$C. All solutions were treated with diethylpyrocarbonate and all glassware was baked to ensure that virus and viral RNA solutions (see below) remained free of RNase.

Alternatively, 1- to 2-month-old C58/J mice were injected with LDV ($10^7$ ID$_{50}$/mouse) by the intraperitoneal route and plasma obtained from the retroorbital sinus at 18, 24 and 42 h after infection was pooled. The plasma was diluted 10-fold with PBS and clarified. Virions were pelleted, gradient-purified, and precipitated by the same methods described above for tissue culture-produced virions. In some experiments [$^3$H]uridine-labelled virions produced in tissue culture were co-purified with the mouse plasma virions and in other experiments, plasma virions were located in gradient fractions spectrophotometrically. In some experiments, the second glycerol gradient was omitted during virion purification.

**Isolation of LDV RNA.** Gradient-purified virions were resuspended in 10 mM-Tris–HCl pH 7.2 containing 2 mM-vanadylribonucleoside complex (Bethesda Research Laboratories) and 2 μg of self-digested Pronase (Sigma). After a 10 min incubation at 37°C, SDS was added to a final concentration of 1% and the solution was
again incubated for 10 min at 37 °C. The extract was twice phenol-extracted and then layered onto a linear 15 to
35% (w/v) sucrose gradient made in a solution composed of 0.05 M-NaCl, 0.01 M-Tris-HCl pH 7.2, 0.001 M-EDTA
and 0.5% (w/v) SDS. Centrifugation was in a SW28 rotor at 24500 r.p.m. for 16 h at 22 °C. Gradient fractions were
collected and aliquots analysed for radioactivity. Fractions containing LDV genome RNA were pooled and the
RNA was precipitated twice with ethanol.

_T1 RNase digestion and 5' end-labelling of oligonucleotides._ Gradient-purified LDV genome RNA was divided
into aliquots and re-precipitated with ethanol. As required, samples of RNA were pelleted, washed twice with
80% ethanol, dried under nitrogen, and resuspended in 8 μl of 10 mM-Tris-HCl pH 7.2, 1 mM-EDTA. The RNA
was digested with RNase T1 (0.8 μg/reaction; Worthington Diagnostic, Freehold, N.J., U.S.A.) at 37 °C for 30
min. After T1 digestion, the 32P-labelled oligonucleotides were extracted twice with phenol and then precipitated
with ethanol. Prior to phenol extraction, oligonucleotides obtained from unlabelled RNA were 5' end-labelled by
incubation with 12 units of polynucleotide kinase (Boehringer-Mannheim) and 150 μCi [γ-32p]ATP (ICN) in 50
mM-Tris-HCl pH 8, 10 mM-MgCl2, 5 mM-DTT, 5% glycerol for 30 min at 37 °C (Simoncits et al., 1977). Precipitated oligonucleotides were electrophoresed in two dimensions according to the method of de Wachter &
Fiers (1972) as described previously (Brinton & Fernandez, 1983).

_3' End-labelling of RNA._ Genome RNA was radio-labelled at its 3' terminus in a reaction mixture containing T4
RNA ligase (Bethesda Research Laboratories) and 32P-pCp according to the method of Keene et al. (1978). The
32P-pCp was prepared using [γ-32p]ATP (ICN), 3'-CMP and polynucleotide kinase (Boehringer-Mannheim).

_Nucleotide sequence analysis._ Aliquots of 3' end-labelled LDV genome RNA were partially digested with either
T1, U2, Bacillus cereus, PhyM, or Cl 3 RNase (Donis-Keller et al., 1977; Lockard et al., 1978; Simoncits et al.,
1977; Boguski et al., 1980) or partially degraded by alkali treatment. The products of the digestions as well as the
alkali ladder were electrophoresed on a 20% sequencing gel (Sanger & Coulson, 1978). Alternatively, samples of 3'
end-labelled RNA were subjected to RNA chemical sequencing reactions performed according to the method of
Peattie (1979).

**RESULTS**

_Oligonucleotide fingerprint patterns of LDV RNAs labelled in vivo and in vitro._

Since no plaque assay for LDV exists, it was not possible to clone the LDV isolates by successive plaque purification prior to analysis of the viral genome RNA by oligonucleotide fingerprinting. Therefore, if virus populations contain more than one virus variant, fingerprints of the RNAs of these populations would be composites. In our initial studies, genome RNA was prepared from virus that was grown in primary C58/J mouse peritoneal macrophage cultures as described in Methods. This genome RNA was uniformly labelled by incorporation of 32P during its replication. Extracellular virions were pelleted, sedimented first on a 10 to 38.5% glycerol–PBS gradient and then on a 20 to 75% glycerol–PBS gradient as described in Methods (Fig. 1a). Subsequently, genome RNA was extracted from the gradient-purified virions and sedimented on a 15 to 30% SDS–sucrose gradient as described in Methods (Fig. 1b). First virions and then viral RNA were gradient-purified to reduce the chance of possible contamination of the LDV RNA with other viral RNAs or cellular RNAs. The sedimentation characteristics of the virions and of the viral RNA were consistent with those previously published for LDV (Brinton-Darnell & Plagemann, 1975). Only a single peak of virion RNA with a sedimentation coefficient of 48S was observed (Fig. 1b). After ethanol precipitation, the 32P-labelled LDV RNA was digested with T1 RNase and subjected to two-dimensional electrophoresis.

The amount of 32P incorporated into LDV RNA during virus replication in macrophage cultures was low. Since only about 5% of the cells in these cultures are susceptible to infection by LDV, the majority of the added 32P, was incorporated into uninfected cells. Actinomycin D was not used to try to improve the labelling efficiency of the viral RNA, because as previously demonstrated (Brinton & Plagemann, 1979) primary murine macrophage cultures are extremely sensitive to actinomycin D toxicity. Due to the low specific activity (2 × 103 to 5 × 103 c.p.m. per 108 LD100 of virus) of the 32P-labelled viral RNA obtained from macrophage cultures, 3 to 4 weeks were required for the exposure of fingerprint autoradiographs. Unique fingerprint patterns were obtained by this method for the LDV-C and LDV-1 genome RNAs (Fig. 2a and b). Although about 100 large oligonucleotides (length greater than 10 nucleotides) were observed in the LDV RNA fingerprints, the number of large RNase T1 oligonucleotides predicted for a 14000 nucleotide RNA could be as high as 200 depending on the base composition and sequence of the RNA (Aaronson et al., 1982).
Fig. 1. Gradient purification of virions and viral RNA. (a) Sedimentation profile of LDV virions on a 20 to 75% (w/v) glycerol-PBS gradient. Virions grown in primary macrophage cultures and labelled with carrier-free $^{32}$P (1 mCi/T75 flask) were pelleted, sedimented on a 10 to 38.5% (w/v) glycerol-PBS gradient and then sedimented through the 20 to 75% glycerol gradient. (b) Virion RNA was extracted and sedimented on a 15 to 30% sucrose-SDS gradient. Brackets indicate fractions that were pooled. Arrows indicate positions of 18S and 28S ribosomal RNA markers.

Fig. 2. Comparison of oligonucleotide fingerprints of LDV genomic RNA labelled by incorporation of $^{32}$P during growth in macrophage cultures. Gradient-purified LDV genomic RNAs were digested with RNase T1 and electrophoresed in two dimensions according to the method of de Wachter & Fiers (1972). The positions of the dye markers xylene cyanol and bromophenol blue are indicated by ×. Autoradiograph exposure time was 42 days (a) LDV-C; (b) LDV-I.
To obtain radioactive oligonucleotides with a higher specific activity for subsequent experiments, oligonucleotides were 5’ end-labelled in vitro. Unlabelled virions were obtained from infected C58 plasma and co-purified with a tracer amount of [3H]uridine-labelled LDV grown in macrophage cultures. The genome RNA was gradient-purified and then digested with T1 RNase. The resulting oligonucleotides were 5’ end-labelled using [γ-32P]ATP and polynucleotide kinase as described in Methods. The fingerprint patterns generated from LDV-1 RNAs labelled in vitro and in vivo were similar (compare Fig. 2b with Fig. 3f). However, some differences were observed between the fingerprint patterns of LDV-C RNAs labelled by the two methods. Four large oligonucleotides (indicated by arrows in Fig. 2a) were observed in the fingerprint of LDV-C labelled in vivo, but were not detected in the fingerprints of the 5’ end-labelled LDV-C RNA (Fig. 3a and 4a). In addition, two spots (indicated by thick arrows in Fig. 4a) were found only in the 5’ end-labelled LDV-C RNA. Differences in the labelling efficiency of particular oligonucleotides during labelling in vivo and in vitro have been previously observed with other RNA virus genomes (Lee & Fowlks, 1982).

Comparison of the oligonucleotide fingerprint patterns of the genomes of six different LDV isolates

Pooled plasma was obtained from groups of 10 C58/J mice infected with 10^7 ID50 of one of six different LDV isolates, LDV-1, LDV-2, LDV-3, LDV-4, LDV-5 or LDV-C. First the virions and then the viral genome RNAs were gradient-purified as described in Methods. Each LDV RNA was then digested with T1 RNase and the resulting oligonucleotides were 5’ end-labelled and electrophoresed in two dimensions (Fig. 3).

The fingerprint pattern obtained for the genomes of each of the six different LDV isolates was distinct. However, comparison of the fingerprint patterns of three of the isolates, LDV-4, LDV-5 and LDV-C (Fig. 4a, b and c) indicated some similarities. Further analysis was carried out using composite fingerprints generated by electrophoresing the oligonucleotides obtained from mixtures of equal amounts of the RNAs of two viruses. Since individual spots have not yet been sequenced, it is not known whether any of the observed spots represent more than one oligonucleotide. Also, a few of the LDV-C oligonucleotides did not appear to be labelled by the in vitro method. For these reasons, the number of observed fingerprint spots may be a low estimate of the actual number of large oligonucleotides. The composite fingerprint of LDV-C and LDV-5 RNAs is shown in Fig. 4(c) and (d). Of the observed 5’-labelled large oligonucleotides, 31 were found only in the RNA of LDV-5 and 21 only in the LDV-C RNA. Fifty-one co-migrating spots were observed. The fingerprints of the LDV-C and LDV-4 RNAs were also compared (data not shown). Five of the 21 spots found only in LDV-C RNA (indicated by arrows in Fig. 4d) co-migrated with spots from the LDV-4 RNA. The composite fingerprint for the RNAs of LDV-4 and LDV-5 is shown in Fig. 4(e) and indicates that these two RNAs share 81 co-migrating spots (Fig. 4e). In addition, the RNA of LDV-4 contained nine spots not found in LDV-5 and the LDV-5 RNA contained 13 spots not found in LDV-4 RNA. Utilizing the statistical confidence limits generated by Aaronson et al. (1982), the degree of genetic relatedness (percentage sequence variation) between these three viruses was estimated. The LDV-4 and LDV-5 RNAs varied from each other by 1.5%, while the LDV-4 and LDV-5 RNAs each varied from LDV-C by 4.5%.

Variability in the efficiency of T1 digestion of one of the LDV-C oligonucleotides was observed (indicated by a double arrow in Fig. 4a). After complete digestion this oligonucleotide migrated at the position indicated by the upper arrow (see Fig. 4a). When digestion was incomplete, this oligonucleotide migrated as a smeared spot at the position indicated by the lower arrow. Sometimes a ladder was seen in this region (Fig. 4a), indicating the possible presence of a region in the RNA with a high degree of secondary structure (Stanley & Vassilenko, 1978; Peattie, 1979; Brinton et al., 1986a).

Effect of passage on the oligonucleotide fingerprint patterns of LDV-1 and LDV-C RNAs

During the experiments described above, the genomic RNA of each isolate was fingerprinted many times. Virus from the same stock pool was used each time an infection was initiated with a
particular isolate to produce viral RNA for fingerprinting. In addition, the amount of virus inoculum and the time of virus harvest were kept constant for each experiment. Under these experimental conditions, the replicate oligonucleotide fingerprint patterns obtained for the genomic RNA of each isolate were identical.

The fingerprints of the genomes of the six LDV isolates shown in Fig. 3 were generated by infecting C58 mice or C58 macrophage cultures with stock virus pools made in Swiss mice (see Methods). The effect of two further C58 passages on the fingerprint patterns of two of the isolates, LDV-C and LDV-1, was subsequently evaluated. As before, C58 macrophage cultures were infected with the stock Swiss mouse pool of LDV-C or LDV-1. The progeny virions were labelled from 4 to 20 h after infection with [3H]uridine added to the medium at a final concentration of 30 μCi/ml. The virions were pelleted from the culture fluid and purified by sedimentation through two successive glycerol gradients as described in Methods. The purified virus obtained from the second gradient was supplemented with foetal calf serum to a final concentration of 10%, and stored at −70 °C. Subsequently, this virus was titrated in mice and then used to infect C58 mice (10^7 ID50/mouse). Pooled plasma obtained at 18 and 24 h after infection was clarified, diluted twofold with PBS and stored at −70 °C. These virus pools were then used to infect C58 mice (10^7 ID50/mouse) to produce genome RNA for fingerprinting. First the virions and then the viral RNA were purified. The RNA was digested with T1 RNase and the resulting oligonucleotides were 5' end-labelled and subjected to fingerprint analysis as described in Methods. The fingerprint patterns of the LDV-1 (data not shown) and LDV-C (Fig. 4f) RNAs obtained after two additional C58 passages were compared to the fingerprints obtained for the same RNA prior to passage (Fig. 3a). Further passage of LDV-C under controlled conditions resulted in the appearance of three or four additional spots (white arrows, Fig. 4f) and the loss of two spots (black arrows, Fig. 4f). The new spot indicated in the lower right hand corner of the fingerprint (Fig. 4f) may represent an incompletely digested oligonucleotide similar to the one indicated in Fig. 4(a). The extent of sequence variation in the LDV-C genome RNA that had occurred during passage was estimated to be 0-375% (Aaronson et al., 1982). In contrast, the fingerprint pattern of the passaged LDV-1 genome RNA (data not shown) was identical to that of the LDV-1 RNA prior to passage (Fig. 3f).

Presence of a 3' poly(A) tract in LDV RNA

The fingerprint patterns obtained with LDV RNAs labelled with 32P, during replication in macrophage cultures clearly indicated the presence of a very large oligonucleotide in the lower left hand corner (Fig. 2). This is the position to which a polynucleotide tract would be expected to migrate. As would be expected, the large oligonucleotide was more efficiently labelled in vivo than by the in vitro end-labelling method (compare Fig. 2 with Fig. 4), but could always be detected when autoradiographs were exposed for longer periods of time.

To determine whether a poly(A) tract was present at the 3' terminus of the LDV RNA, direct RNA sequencing of 3' end-labelled LDV RNA was carried out. Purified LDV RNA was labelled at its 3' terminus using 32P-pCp and T4-RNA ligase and then subjected to partial RNase digestion with T1, U2 or PhyM RNase (T1 cuts at Gs, U2 cuts at As, and PhyM cuts at As and Us) or to chemical modification according to the method of Peattie (1979) as described in Methods. A poly(A) region of more than 50 nucleotides was found at the 3' terminus of the LDV RNA (Fig. 5).
Genotypic variation of LDVs

Fig. 5. Autoradiograph of 3’ end-labelled LDV genomic RNA subjected to (a) chemical modification sequencing (Peattie, 1979) or (b) partial enzymic hydrolysis sequencing using RNases T1, U2 and PhyM. RNA fragments were electrophoresed on 20% sequencing gels (Sanger & Coulson, 1978). BH, base hydrolysis ladder. The positions of marker dyes xylene cyanol FF and bromophenol blue are indicated.

Fig. 4. Comparison of the oligonucleotide fingerprints of the genome RNAs of LDV-C, LDV-4 and LDV-5. Genome RNAs were purified from virions obtained from C58 mouse plasma and the oligonucleotides were 5’ end-labelled after digestion with T1 RNase. (a) LDV-C, (b) LDV-5, (c) a mixture of equal amounts of LDV-5 and LDV-C and (d) drawing of the panel (c) composite fingerprint. Black spots are those found only in LDV-5 RNA; dark grey spots are those found only in LDV-C RNA. Arrows indicate spots that co-migrated in LDV-4 and LDV-C RNAs. (e) Drawing of the composite oligonucleotide fingerprint generated from a mixture of LDV-4 and LDV-5 RNAs. Black spots indicate oligonucleotide spots found only in LDV-5; dark grey spots indicate oligonucleotides unique to LDV-4. (f) Effect of virus passage on the oligonucleotide fingerprint of LDV-C genomic RNA. The LDV-C was passaged in C58 mice, then in C58 macrophage cultures and again in C58 mice. Genome RNA was purified from the last mouse passage and fingerprinted. White arrows indicate new spots appearing in LDV-C genomic RNA after passage; black arrows indicate position of lost spots.
DISCUSSION

Whereas data from two previous studies (Bailey et al., 1965; Cafruny & Plagemann, 1982) had indicated some antigenic diversity among LDV isolates, extensive analysis of LDV isolates by classical serological techniques has not been technically feasible. Oligonucleotide fingerprinting offers an alternative means for further characterization of LDV isolates. The value of this technique for subtyping LDV isolates was indicated by our demonstration of a close genetic relationship (1.5% variation) between LDV-4 and LDV-5 which were subsequently found to have been derived from the same virus stock at different passage levels (see Methods).

It has previously been reported that differences in fingerprint spot intensities are sometimes observed with in vitro and in vivo oligonucleotide-labelling techniques (Lee & Fowlks, 1982). Although a few of the largest LDV-C oligonucleotides were not detected after in vitro labelling, the in vitro labelling technique was preferable because of the higher specific activity of the oligonucleotides obtained by this method. Two of the LDV-C fingerprint spots were observed only in fingerprints of RNA labelled in vitro (Fig. 4a). One possible explanation for the appearance of these spots is that they are generated from the missing large oligonucleotides by cleavage at G residues that are difficult to digest. Incubation of the RNA with RNase T1 is twice as long in the in vitro labelling method as in the in vivo method. Since the same spot differences were observed in fingerprints of in vivo and in vitro labelled LDV-C RNA in several experiments, it seems unlikely that these two spots were due to a random contamination with another viral or cellular RNA. Variable low density spots were occasionally observed in RNA preparations labelled in vitro and could represent a low level of contaminating RNA or LDV oligonucleotides generated by digestion with other nucleases during RNA purification.

Although we have not been able to document a known historical connection between LDV-C and the isolate from which LDV-4 and LDV-5 were derived, the relatively low degree (4.5%) of sequence variation between these LDV isolates suggests the possibility of a common origin. The LDV-C isolate was originally obtained from a line of Ib leukaemia cells (Martinez et al., 1980) derived from a 1 year old C58 mouse in 1929 (MacDowell & Richter, 1932) and was maintained for many years by serial passage in young (less than 6 months of age) C58 mice (Murphy et al., 1970). It is not known when the Ib leukaemia cell suspensions became contaminated with a passenger LDV, but presumably one or more of the recipient C58 mice in which the Ib leukaemia cells were passaged was already infected with LDV. Thereafter, all mice injected with the Ib leukaemia cell suspensions also became infected with LDV. The LDV which initially contaminated the Ib leukaemia cell suspensions may have been phenotypically similar to LDV-4, but prolonged passage of this virus in C58 mice may have resulted in a subsequent amplification of a variant subpopulation with an increased ability to infect ventral horn motor neurons, a second target cell for LDV infection in C58 mice which are susceptible to LDV-induced poliomyelitis (Stroop et al., 1985; Brinton et al., 1986b).

Some of the LDV isolates appear to be more stable during passage than others. Our preliminary results show that two acute infection passages carried out under standard conditions in C58 mice can result in a number of changes in the oligonucleotide fingerprint pattern of LDV-C. Interestingly, the RNAs of LDV-4 and LDV-5 also clearly demonstrate that rapid genetic change can occur during passage. Unfortunately, we could not ascertain with certainty from old records the strain of mice, the amount of virus inoculum, or the time of virus harvest that had been used for the passages separating LDV-4 and LDV-5. In contrast, the fingerprint pattern of LDV-1 remained unchanged during two passages. The high degree of genetic variability observed for the related LDV-C, LDV-4, and LDV-5 viruses during passage could be due to the presence of hypervariable regions in their genomes. Alternatively, these isolates may represent mixtures of two or more virus variants that are selectively amplified during passage. Under the experimental conditions utilized for acute infection passage (virus was harvested by 48 h after infection), antiviral antibody would play no role in a possible selection of virus subpopulations. Further analysis of the LDV genome will be necessary before the greater genetic variability observed for the related LDV-C, LDV-4, and LDV-5 isolates can be explained. The knowledge that this genome variability exists is important for future studies involving cloning and sequencing of LDV genomes.
The demonstration that the LDV genome has a 3'-terminal poly(A) sequence has relevance for virus classification. Previously LDV had been thought to be more closely related to the flaviviruses than to alpha togaviruses (Brinton-Darnell & Plagemann, 1975). However, since flaviviruses have no 3' poly(A) (Westaway et al., 1985), the presence of 3' poly(A) on the LDV genome distinguishes it from the flaviviruses. Further characterization of the LDV genome structure and its gene order are necessary before the extent of similarity between togaviruses and LDV can be assessed.

Our data indicate that oligonucleotide fingerprint analysis is a useful method for subtyping LDV isolates. Additional LDV isolates now need to be analysed in order to determine the number of LDV genetic subtypes which exist. Several LDV isolates have been obtained directly from wild mice in Australia (Pope, 1961), Germany (Georgii & Kirschenhofer, 1965), the United States (Pope & Rowe, 1964) and England (Rowson, 1963; Field & Adams, 1968). It will be of interest to compare the fingerprints of genomic RNAs of isolates from different geographical areas with those obtained from laboratory mice.

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