Lymphotropic Strain SL3 of Aleutian Disease Virus: Identification of Replicative Form DNA, Molecular Cloning and Expression of Capsid-specific Proteins

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SUMMARY

Replicative form (RF) DNA of the lymphotropic strain SL3 of Aleutian disease virus was isolated from infected cell cultures. A novel intermediate of about 7.6 kilobases was demonstrated in Hirt lysates in addition to single-stranded viral, double-stranded monomer and dimer RF DNA. The monomer RF DNA exhibited a length heterogeneity of 70 bp and 160 bp at its 3' and 5' termini. The two major monomer RF DNA species each contained hairpins in the extended or the foldback configurations. A central fragment between map units 0.15 and 0.88 was cloned into plasmid pUC18. The recombinant clone expressed virus-specific proteins ranging from 32000 to 74000 mol. wt.

INTRODUCTION

Aleutian disease (AD), a persistent infection in non-Aleutian genotype mink, is caused by an autonomous parvovirus, the Aleutian disease virus (ADV) (Bloom et al., 1980). As a characteristic of this virus group (Siegl et al., 1985), ADV contains a linear, 4.8 kb ssDNA of negative polarity (Bloom et al., 1980). After infection, this DNA is converted into ds monomer and dimer replicative form (RF) DNA. These three DNA species have been identified in Hirt supernatants of ADV variant Gorham (ADV-G)-infected cell cultures. The monomer RF DNA corresponds to the unit length genome, whereas the dimer RF DNA consists of two linked genome equivalents (Bloom et al., 1983). It has been demonstrated for minute virus of mice (MVM) that both dsDNAs are used for the synthesis of progeny ssDNA (Ward & Dadachanji, 1978).

The ADV particle consists of two major structural proteins of 85000 (85K) and 75K mol. wt., which share extensive antigenic cross-reactivity. A third ADV-encoded protein of 71K is antigenically distinct (Bloom et al., 1982; Kierek-Jaszczuk et al., 1986). As demonstrated for MVM (Labieniec-Pintel & Pintel, 1986; Astell et al., 1983) and H-1 parvovirus (Rhode & Paradiso, 1983), the structural proteins are transcribed from the right side of the genome, whereas the left side codes for the non-structural protein(s). To date, nothing is known about the function(s) of the non-structural protein, but it is considered to be involved in DNA replication or ssDNA synthesis (Hauswirth, 1984).

A physical map of the ADV-G genome has been published by Bloom et al. (1983) and the central 80% of the genome has been cloned in three non-overlapping fragments (Mayer et al., 1983). A recombinant clone harbouring the right part of the genome expressed ADV-specific proteins which were considered to be capsid-specific.

In this paper we report on the RF DNA of the ADV isolate SL3 (van Dawen et al., 1983), which shows a marked lymphotropism in vivo and in vitro (Roth et al., 1984; Kaaden et al., 1986). The virus genome was physically mapped and the monomer RF DNA characterized with emphasis on its terminal structures. A central genome fragment (73%) was cloned into the expression vector pUC18 and capsid-specific proteins were demonstrated by Western blot techniques with monoclonal antibodies (MAbs).
**Methods**

**Cells and virus.** CCC clone 81 cells (Sliski et al., 1977) were infected with ADV-SL3 at an m.o.i. of 10 (van Dawen et al., 1983). The absence of mink enteritis virus in stock virus was proven by dot blots, using a cloned DNA fragment of feline panleukopenia virus (FPV) as a probe. The FPV clone with the insert from map units (m.u.) 20 to 59 was a gift from Colin Parrish.

**Isolation of ADV RF DNA.** Infected cells were lysed 48 h post-infection (p.i.), since at this time the highest yield of viral DNA was obtained. Modified Hirt extractions (Hirt, 1967) were performed (Kaaden et al., 1986). The phenol-extracted and purified DNA was separated on 0.8 % agarose gels (NA-agarose, Pharmacia) in Tris-acetate-EDTA buffer (Maniatis et al., 1982). Monomer RF DNA was extracted from preparative gels as previously reported (Kaaden et al., 1986).

**Isolation of virion DNA.** Infected cells were harvested 96 h p.i. and processed by freezing and thawing. An equal volume of a saturated ammonium sulphate solution pH 9.5 was added to the clarified lysate and precipitated at 4 °C overnight. The precipitate was collected by centrifugation at 14 800 g for 40 min at 4 °C. It was resuspended in 10 mM-Tris-HCl pH 7.4, 1 mM-2-mercaptoethanol, 3 mM-SDS, 100 &g/ml proteinase K (Boehringer). Proteinase K was inactivated by addition of 20 mM-EDTA (final concentration) and DNAase (40 &g/ml, Boehringer). DNAase was inactivated by addition of 20 mM-EDTA (final concentration) and heating for 10 min at 60 °C. The viral DNA was released for 30 min at 37 °C in the presence of 0.6 % SDS and 75 &g/ml proteinase K (Boehringer), phenol-extracted and precipitated with ethanol.

**DNA techniques.** Purified ADV monomer DNA was labelled with [35S]- or [32P]-dCTP as previously described (Kaaden et al., 1986). Transfer of electrophoretically separated DNA onto filters was performed according to Southern (1975) with 20 & SSC buffer (SSC is 150 mM-NaCl, 15 mM-sodium citrate) onto nitrocellulose membranes (BAB5, Schleicher & Schuell) or with 10 & SSC onto Zeta-Probe membranes (Bio-Rad). DNA was fragmented prior to blotting by partial depurination for 9 min in 0.25 M-HCl (Meinkoth & Wahl, 1984). Hybridization was according to Wahl et al. (1979). Nitrocellulose filters were exposed to DEF-2 X-ray film (Kodak) at -70 °C in a Cronex Cassette (DuPont). Restriction endonucleases were used as recommended by the suppliers except for double digestion with HindIII and BamHI (Boehringer), which was done in 10 mM-Tris-HCl pH 7.4, 1 mM-dithioerythritol and 50 mM-NaCl (Maniatis et al., 1982). S1 nuclease (Bethesda Research Laboratories, BRL) digests were done essentially as described by Favaloro et al. (1980) at 37 °C.

**Molecular cloning.** Escherichia coli JMI09 (Yanisch-Perron et al., 1985) was used as hosts for plasmid pUC18 (Norrander et al., 1983) were grown on NZY minimal agar plates (Miller, 1972). Transformed bacteria were maintained in NZY medium (BRL) with 50 &g/ml ampicillin (Bayer). For ligation, double-digested plasmid and insert DNA were mixed in a molecular ratio of 3:1 in 90 mM-Tris-HCl pH 8.0, 10 mM-MgCl2. The mixture was heated at 65 °C for 5 min and DNA was precipitated with ethanol in the presence of 2.5 M-ammonium acetate. Ligation of annealed DNA was performed with a vector concentration of 1 &g/ml (Maniatis et al., 1982).

**Transformation of JMI09 cells.** JMI09 cells was done as described (Hanahan, 1983) except that dimethylformamide and dithiothreitol were used. Recombinants were screened on NZY plates containing 0.2 &g/ml IPTG (isopropylthio-β-D-galactoside) and 40 &g/ml XGal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Boehringer). Replicas of colourless colonies were transferred onto nitrocellulose filters (HATF membrane, Millipore). In an additional step of the colony hybridization procedure of Grunstein & Hogness (1975), the filters were equilibrated for 6 min in 0.3 M-NaCl, covered with 0.9 ml of proteinase K (1-2 mg/ml in Tris-EDTA buffer) and incubated for 1 h at 42 °C. ADV-positive clones were identified by DNA hybridization and cloned twice.

**Plasmid DNA was extracted according to Ish-Horowicz & Burke (1981) and twice purified in ethidium bromide (EtBr)-containing CsCl gradients. EtBr was extracted and the DNA precipitated with ethanol after 1:4 dilution (Maniatis et al., 1982). Plasmid pBM1 (Mayer et al., 1983), containing ADV-G sequences between m.u. 0-54 and 0-88 was provided by Marshall E. Bloom.

**Expression and detection of virus-specific proteins.** Recombinant bacteria and control cells were grown in 5 ml NZY to an OD$_{560}$ of 0.5. The expression of lac operon-dependent proteins was induced with 0-2 mM-IPTG (Miller, 1972) after plasmid amplification with chloramphenicol (Neidhardt et al., 1980). The bacteria were suspended in 600 μl 62.5 mM-Tris-HCl pH 8.0, 5.5 % 2-mercaptoethanol, 3 % SDS, 1 mM-phenylmethylsulphonyl fluoride (Merek) and boiled for 5 min. The protein concentration was determined according to Schaffner & Weissmann (1979) and 50 μg protein samples were applied to 10 % PAGE gels (Laemmli, 1970). Electrophoresis was for 5 h at 20 mA. Cell extracts containing the virus-specific antigens p85, p75 and p71 (Kierzek-Jaszczuk & Kaaden, 1986) were used as controls for the immune reaction. A pre-stained high mol. wt. protein standard mixture (BRL) was included for mol. wt. determination. Protein transfer (Towbin et al., 1979) onto nitrocellulose sheets was at 35 V, 12 °C for 90 min in a LKB blot chamber. The transfer buffer contained no methanol. The immune reaction with the MAbs was done as previously described (Kierzek-Jaszczuk et al., 1986).
RESULTS
Isolation and identification of ADV DNA

ADV- and mock-infected cells were lysed 48 h p.i., Hirt-extracted and aliquots of both DNA samples were electrophoretically separated as described. After staining with EtBr, two bands of 9.6 and 2.4 kb and one double band of 4.8 kb were detected in DNA preparations from ADV-infected cells (data not shown). By analogy to published findings for ADV RF DNA (Mayer et al., 1983), the double band was assumed to correspond to the ADV monomer RF DNA and the 9.6 kb to the dimer RF DNA. The fastest migrating band in extracts of infected cells was assumed to be viral ssDNA.

Hybridization was performed for the specific identification of these DNA bands. The ADV monomer RF DNA of 4.8 kb was purified, labelled by nick translation with [35S]dCTP and used as a probe. Hirt lysates from ADV- and mock-infected cells, ADV monomer RF DNA and viral ssDNA from infectious particles were prepared as described. DNA samples were run on 0.8% agarose gels, transferred to nitrocellulose membranes and probed. The bands identified after autoradiography are shown in Fig. 1. The probe hybridized specifically with the purified viral ssDNA, which migrated under non-denaturing conditions with a length of 2.4 kb (lane 3). This band was also detected in Hirt extracts of infected cells (lane 2). In the ssDNA sample, an additional band of 4.8 kb, comigrating with the monomer RF DNA (lane 4), was demonstrated. As expected, the double band at 4.8 kb gave strong signals, in addition to the 9.6 kb band. A fourth DNA species was detected in Hirt extracts, migrating at approximately 7.6 kb. No specific hybridization was shown in DNA preparations originating from mock-infected cells (lane 1). The specificity of the identified DNA species was further confirmed by Southern blot hybridization using the cloned pBM1 fragment of ADV-G DNA as a probe (data not shown).

To determine whether ss regions were present in the RF DNA, Hirt lysates were digested with S1 nuclease. Undigested lysates and samples, withdrawn after 10, 20, 40 and 60 min of incubation, were electrophoretically separated and transferred onto nitrocellulose membranes. After hybridization (data not shown), no viral ssDNA or 7-6 kb DNA was detected in any of the S1 nuclease-treated samples. In the control lane all ADV DNA species described were present.

Restriction analysis of ADV monomer RF DNA

After cleavage of the DNA with restriction endonucleases, the fragments were separated on 1.4% agarose gels. The following restriction endonucleases had no cleavage sites on the ADV monomer RF DNA: BglI, BglII, ClaI, SacI and SalI. Endonuclease PstI cleaved at m.u. 0.07, BamHI at 0.15, TaqI at 0.41, EcoRI at 0.54, EcoRV at 0.68, and HindIII at m.u. 0.88. The localization of the 3' and 5' termini was derived from Bloom et al. (1983) as no difference in the restriction pattern compared to the ADV-G variant was observed. The smaller fragments were consistently separated as a double band. This length heterogeneity was approximately 160 bp for the 5'-terminal HindIII or EcoRV fragments; the small 3'-terminal fragments of the BamHI- and PstI-cleaved ADV monomer RF DNA differed by about 70 bp. For further confirmation, we cleaved 0.1 μg of monomer RF DNA with HindIII or BamHI or with both enzymes simultaneously. After Southern blotting onto Zeta-Probe membranes, heterogeneity of the 3' terminus after BamHI digestion and the 5' heterogeneity after HindIII cleavage was demonstrated (Fig. 2, lanes 2 and 3). As expected, in lane 4 representing the double digest, both pairs of double bands were detected.

Molecular cloning of ADV monomer RF DNA

A forced cloning strategy was used to introduce the central fragment of the ADV monomer RF DNA from m.u. 0.15 (BamHI site) to m.u. 0.88 (HindIII site) into the multiple cloning site (MCS) of plasmid pUC18 (Fig. 3). HindIII- and BamHI-digested ADV monomer DNA was electrophoretically separated and the 3.5 kb band, representing the central fragment, was extracted from the gel slice. The excised DNA sequence of the plasmid polylinker was removed by ethanol precipitation.

After transformation of competent cells with the ADV-pUC18 recombinant DNA, colourless colonies were screened. Only clones containing an excisable ADV-specific fragment of 3.5 kb
Fig. 1. Identification of ADV-specific DNA in Hirt extracts and purified virions in the presence of cloned ADV DNA and purified monomer RF DNA by the Southern blot technique. Lane 1, Hirt extract of mock-infected clone 81 cells 48 h p.i.; lane 2, Hirt extract of ADV-infected clone 81 cells 48 h p.i.; lane 3, ssDNA from infectious ADV particles; lane 4, purified ADV monomer RF DNA; lane 5, plasmid pSL18 cleaved with HindIII and BamHI.

Fig. 2. Demonstration of the terminal length heterogeneity of ADV monomer RF DNA (lane 1) digested with BamHI (lane 2), HindIII (lane 3) or both enzymes simultaneously (lane 4). The fragments were separated on a 1.4% agarose gel, blotted onto Zeta-Probe membranes and hybridized against $^{32}$P-labelled ADV monomer RF DNA. The asterisks indicate the position of the terminal fragments. Completely and partially digested DNA of higher mol. wt. was not resolved in the 1.4% gel.

were further processed. The specificity of the excised insert was confirmed by length determination with BamHI- and HindIII-digested ADV monomer RF DNA (data not shown) and Southern blot hybridization (Fig. 1, lane 5) in the presence of viral and RF DNA (lanes 3 and 2). $^{35}$S-labelled ADV monomer RF DNA was used as a probe.

The clone with the ADV DNA insert was designated pSL18. The insert was extracted and purified from the recombinant plasmid by batch preparation as mentioned above.

Detection of protein expression in recombinant clones.

Because of the cloning strategy applied (Fig. 3), the expression of a fusion protein, harbouring virus-specific sequences, was tested. JM109 cells containing or lacking plasmid pUC18 and the bacterial clone pSL18 were grown and induced for protein expression. Fifty μg samples of bacterial proteins were separated by PAGE, blotted and screened with MAbs as described.
Characterization and cloning of ADV-SL3 DNA

Fig. 3. Cloning strategy for the central 73% of the ADV genome between the BamHI and HindIII cleavage sites (upper bar, localization of the restriction sites is indicated by their corresponding m.u.) and their positions in relation to the 3' or 5' terminus of the genome. The lower graphic represents the orientation of the insert in the MCS relative to the lac operon promoter (lac) and the direction of transcription (indicated by an arrow) for the recombinant plasmid pSL18.

Fig. 4. Identification of expressed capsid-specific proteins in induced recombinant pSL18. JM109 cells, lacking or harbouring pUC18 (lane 3 or 4, respectively), or containing the recombinant plasmid pSL18 (lane 2) were screened for virus-specific protein expression. In addition, a crude ADV-specific antigen extract (lane 1) was separated by PAGE, transferred onto a nitrocellulose filter and screened with the MAb pool (see text). A specific reaction was obtained with the ADV-antigens p85 and p75 and proteins expressed from pSL18 ranging from 32K to 74K.

MAb2, specific for the non-structural ADV protein p71, and a pool consisting of MAbs 47, 66, 77 and 84 recognizing common epitopes on the virus capsid proteins p75 and p85 (Kierck-Jaszczuk et al., 1986) were used. Fifty µg of a crude extract of ADV-infected clone 81 cells and 120 µg of a prestained protein mixture were run in parallel. The immune reaction revealed that none of the MAbs reacted with proteins expressed in JM109 harbouring or lacking plasmid pUC18. The MAb pool (Fig. 4), specific for the capsid components p75 and p85 (lane 1), recognized virus-specific proteins in clone pSL18 (lane 2). The different capsid-specific proteins ranged from 32K to 74K, the major bands migrated at 32K and 54K. MAb2 (data not shown) did not detect ADV-induced proteins from pSL18.

DISCUSSION

All investigations presented in this paper were done with DNA derived from the ADV isolate SL3. It originates from the bone marrow of a naturally infected mink and has been adapted to grow in cell culture (van Dawen et al., 1983). ADV-SL3 exhibits a strong lymphotropism in vivo and in vitro. ADV-specific antigen has been detected in mononuclear cells isolated from naturally infected mink (Roth et al., 1984) and virus replication can be demonstrated in vitro by infecting enriched T and B lymphocytes derived from ADV-negative mink (Kaaden et al., 1986).
ADV-specific DNA bands were only detected in Hirt supernatants from virus-infected cells. ADV-specific signals were obtained with viral DNA from infectious particles and Hirt supernatants. Whether the width of the viral ssDNA band is due to some degradation or whether it reflects a heterogeneity of the viral DNA is not yet known. Monomer and dimer RF DNA species (Bloom et al., 1983) of 4·8 and 9·6 kb, respectively, were also detected. The ADV-specific DNA species of 7·6 kb, however, has not yet been described for ADV. The S1 nuclease sensitivity of this DNA species indicates that extensive ss regions are present. These features have been reported for a partially replicated dimer intermediate of MVM, migrating with a similar length (Faust & Gloor, 1984). The virus-specific band of 4·8 kb in the virion DNA preparation, comigrating with monomer DNA under non-denaturing conditions, can be assumed to represent annealed plus- and minus-stranded ssDNA. Contamination with RF DNA can be excluded by the results of extensive incubation of the purified virions with DNase.

The encapsidation of varying quantities of plus strands has already been reported for LuIII, H-1 parvovirus, Kilham rat virus (Bates et al., 1984) and MVM (Bourguignon et al., 1976). ADV monomer RF DNA exhibits a length heterogeneity, characteristic of other parvoviruses (Hauswirth, 1984). This is most readily detectable amongst terminal restriction fragments as described for MVM (Ward & Dadachanji, 1978; Astell et al., 1985) and H-1 parvovirus (Rhode, 1977). The finding that the monomer RF DNA migrates as a double band (Fig. 1, lane 4) has been described for ADV (Mayer et al., 1983) but not for other parvoviruses. Southern blot hybridization with cloned ADV DNA (data not shown) demonstrated that the monomer RF double band consists only of ADV-specific DNA. The specificity of the terminal restriction fragments is confirmed by hybridization with labelled monomer RF DNA. The length heterogeneity of the 5' terminus is 160 bp, a value 60 bp higher than that reported by Bloom et al. (1983). The 3' terminus fragments differ by about 70 bp. The sum of both values results in a maximum length difference of 230 bp for the monomer RF DNA. This value is very close to the observed difference of 250 bp for the two bands of ADV monomer RF DNA. By analogy with MVM (Ward & Dadachanji, 1978) this implies that in the slower migrating DNA both termini have extended hairpins. The palindromes of the faster migrating component exhibit the foldback configuration with a covalent linkage between the DNA strands. This DNA species is only detectable in Southern blots if the DNA has been depurinated prior to blotting. After this step the DNA is fragmented, and therefore no intramolecular reassociation can occur. Likewise, depurination is necessary to detect the faster migrating terminal restriction fragments by hybridization. The relative proportions of these monomer RF species also varies as observed for MVM (Chow et al., 1986). All ADV RF DNA species are under further investigation at present.

A strategy was used to clone the central 3·5 kb genome fragment of ADV-SL3 into the expression vector pUC18 such that lac operon-directed transcription started from the left end of the viral insert. It was shown for MVM (Labieniec-Pintel & Pintel, 1986; Astell et al., 1983) or H-1 parvoviruses (Rhode & Paradiso, 1983) that transcription exclusively proceeds in this direction. In all parvovirus systems investigated so far, the left side of the genome codes for the non-structural and the right part for the capsid proteins. The finding of Mayer et al. (1983) who demonstrated the expression of a probably capsid-specific protein by a cloned ADV insert spanning m.u. 0·55 to 0·88 parallels these findings and suggests that the genome organization of ADV is similar to that described for other parvoviruses (Carter et al., 1984).

Our results support the direction of transcription, whereas the expression of capsid-specific proteins starting at m.u. 0·15 is contradictory to the reported results. The great number of distinct ADV-specific protein bands is probably a result of proteolytic degradation of unstable precursor molecule(s) as observed by Goeddel et al. (1979) for the lac operon-dependent expression of human growth hormone in E. coli. A possible genetic rearrangement translocating capsid-specific DNA sequences was excluded by the results of restriction digestion of pSL18 DNA with TaqI. By these experiments (data not shown) we confirmed that the orientation of the insert is correct and that the restriction pattern of the cloned DNA is not altered. Considering our experimental data we postulate that an unknown mechanism allows the expression of ADV DNA sequences located far downstream from the lac promoter. Sequence analysis and subcloning of ADV DNA into other expression vectors are in progress to prove this hypothesis.
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