C5-691

Progress report (June 1, 1989 - December 31, 1989) on the grant # 5544-G-SS-7006-00 "Cloning and Expression of Genes of Newcastle Disease Virus"

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The previous report covered the experiments on recloning the candidate insert from the phage recombinant B27 into Bluescript plasmid vectors, sequencing its 3' end (mRNA sense), identifying it as practically full-length cDNA equivalent of the NDV HN gene, developing a cDNA diagnostic probe and developing a strategy for DNA modifications with the aim to make them suitable for expression.

The current report describes modification of both the HN cDNA and expression vector, an attempt to express the construct, improving the protocol for probing the virus with cloned cDNA and an unexpected finding - a unique 20 base 3'-terminal HN sequence,

MATERIALS AND METHODS

Materials. All the restriction and DNA-modifying enzymes were purchased from Boehringer Manheim, chemicals - from Sigma, radioactive precursors - from Amersham.

Single-stranded DNA (ssDNA) isolation. Individual DNA strands were rescued from the plasmid recombinants by M13 helper phage, strain R408 (UBS), according to the manufacturer's protocol.

Sequencing ssDNA's. The rescued ssDNA's (or purified dsDNA's) were sequenced by chain termination method of Sanger et al. (1977) with 32P- or 35S-dATP. The preparations were electrophoresed in 8% polyacrylamide gels containing 9M urea and exposed to X-ray films for autoradiography.

Computer analysis. All the computations have been done with the NIH sequencing package, NIH MBUG, ver. 87.1.

RESULTS

Modification of the HN cDNA and expression vector for recombining. The 2-step modification of the HN cDNA and 2-step modification of the SK Bluescript expression vector have been done according to the strategy outlined in the previous report. The modified cDNA (containing one blunt and one HindIII ends) was ligated to the modified vector (containing one blunt and one HindIII ends), and the resulting preparation was used for transforming an appropriate host. Analysis of resulting colonies revealed presence of a number recombinant plasmids that contained 1.7 kbp or shorter inserts (not shown). No desired inserts (2 kbp long) have so far been found. This, we believe, is because the HN cDNA contains 8 HaeIII recognition sequences (positions 21, 355, 926, 979, 1019, 1153, 1288, 1437), and fishing out the desired fragment with the only cut in the first (position 21) restriction site is tricky. None of the isolated recombinants can be expressed since - according to the computer analysis of each of them - only the first HaeIII cut generates a proper fragment for the in-frame insertion. We are in the process of repeating the experiment that includes two time-consuming controls for:

1. Partial HaeIII digestion of the cDNA;

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2. Mung Bean nuclease digestion of the EcoRI-digested vector.

Developing a cDNA diagnostic probe. The KSP47 recombinant DNA that carries 2 kbp NDV-specific HN cDNA sequence (see the previous report) has been used for probing NDV, and the result is shown in Fig. 1. The protocol for blotting the virus has been modified: the virus was disintegrated by an ionic detergent rather than by alkali in order to prevent viral RNA from alkaline hydrolysis. Three conclusions can be made from the Figure:

i. viral genomes (RNA) are blotted without prior extraction of the nucleic acid which is crucial for diagnostic purposes (rows A and B);

ii. The probe specifically hybridizes to the NDV RNA (no signal with unrelated influenza RNA is seen in the rows "C");

iii. as little as 3000 pfu of NDV give a clear positive signal with the probe;

Thus, a NDV-specific cDNA that can be utilized for specific identification of the virus in the capacity of a cDNA diagnostic probe has been developed. Utilization of the probe for diagnostics requires, however, some additional experiments (see "Discussion").

Unique 3'-terminal sequence of the HN cDNA. In order to confirm that the cloned HN cDNA contains the entire open reading frame (orf) for the HN polypeptide, the 3' terminus of the cDNA (mRNA sense) has been sequenced (Fig. 2) in addition to its 5' end (which has been shown in the previous report). Though some background lines are seen at the Figure, the gel is readable. Comparison of the sequence from Fig. 2 to the reference HN sequence (Fig. 3) shows that the 3' HN terminus is intact in the cloned cDNA as well, i.e. the cloned cDNA contains the entire orf for the HN glycoprotein. However, the Figure also reveals an unexpected and intriguing finding: the cloned cDNA not only contains the entire 3' reference terminal sequence, but also additional 20 bases that are absent from the reference HN cDNA (Fig. 3, A). These 20 bases form the very 3' terminus in the Australia-Victoria HN gene which reveals a high degree of homology to the 5' terminus of the downstream L gene (Fig. 3, B). This finding will be discussed below in more detail.

DISCUSSION

Developing a cDNA diagnostic probe. Successful utilization of a clone containing the HN-specific sequence for probing the NDV genomes is demonstrated in Fig. 1 where blotted NDV (rows A and B), but not influenza virus (row C) reveal clear hybridization signals. Thus, one of the aims of the project has been achieved: the synthesized and cloned cDNA specifically recognizes NDV genomes without their prior isolation from virions. The result presented in the Fig. 1 was obtained after disintegration of the virus with an ionic detergent rather than with alkali (glyoxal was added for stretching out the released RNA). Evidently, optimal conditions for probing the virus must be elaborated prior to utilization of the probe for direct diagnostics. These must include comparisons of different tissue or organ sources (blood, organ homogenates, swabs, etc), different ways of blotting as well as different kinds of membranes for immobilization of released genomes. If a membrane that binds RNA and not proteins (or vice versa) is found, a

significant increase in sensitivity could be expected: all the immobilized RNA might become accessible for the probe in this case.

3' terminus of the HN cDNA. Both 5' and 3' termini of the NDV genes contain consensus sequences that are either identical or very similar for all the genes (Millar et al., 1986). 3' consensus sequences supposedly secure posttranscriptional adenylation of mRNA's and consist of TAAG followed by 5-6 adenylate residues. The cDNA sequence which is shown in Fig. 2 contains two TTG repeats which replace the AAAAAA (the latter is not shown in the reference sequence, Fig. 3, A, upper row, but is shown in the Fig. 3, B). The terminal AAAAAA in the cDNA sequenced (Fig. 2) is absent probably because the oligo (dT) primer was not ligated to the 1st strand cDNA synthesized (the ligase was not present in the assay during the cDNA synthesis step). However, it is clear from the sequence shown that the terminal TAAG (reference sequence) is replaced by the terminal CAAG (cDNA). Moreover, the entire 3' terminus in the cDNA synthesized has rather high degree of homology to the 5' end of the downstream L gene. One could suggest that this sequence belongs to the L gene which in the case of Australia-Victoria is not identical to that of Beaudette. The suggestion seems unlikely for the following reasons:

i. the cDNA was synthesized on poly(A)-RNA's, and there is no (A)n that immediately follows the 5'-AAGTGGCAAT-3' of the L to which the oligo (dT) primer could theoretically be annealed (if either L-HN dicistronic mRNA or antigenomic viral RNA were utilized by the enzyme as templates).

ii. the sequence lacks the (A)5-6 adenylation site that would follow GATTAAG, if synthesis starts in the L.

iii. the sequence contains additional 6 bases (two TTG repeats) that are absent from the L sequence.

Thus, sequencing of the HN cDNA synthesized and cloned conclusively identifies it as practically full-length HN sequence and reveals presence of unknown 20 base 3' terminus with high degree of homology to the 5' terminus of the downstream L gene. It is hard to speculate on possible function of this sequence without further analysis of the situation which is an independent project by itself.

Publications (in the framework of the current grant):

1. V. Zaslavsky and T. Molad. 1990. A possible approach to enrich cDNA yields with full-length molecules. Virus Genes, in press.

2. T. Molad and V. Zaslavsky. Improved analytical isolation of bacterial plasmids". DNA and Protein Engineering Techniques. Submitted on Nov. 9, 1989.

LEGENDS TO THE FIGURES

Fig. 1. Probing of NDV with KSP47 Purified ($2x10^4$ HA, $2x10^{10}$ pfu/ml) and allantoic ($2x10^2$ HA, $2x10^8$ pfu/ml) NDV as well as allantoic influenza virus ($2x10^2$ HA) have been treated (50 C, 1 hr) with equal volumes of PBS containing 2 M glyoxal and 1% SDS. 2-fold dilutions of the viruses have been made in 0.5% SDS. 10 ul of each dilution (in duplicates) have been applied to a nitrocellulose membrane in a blotting apparatus, washed with 100 ul of 0.5% SDS, dried, and hybridized to a NDV-specific cDNA recombinant labeled by 32P (10^6 cpm/ml of the hybridization mixture). A, purified NDV. B, allantoic NDV. C, allantoic influenza virus;

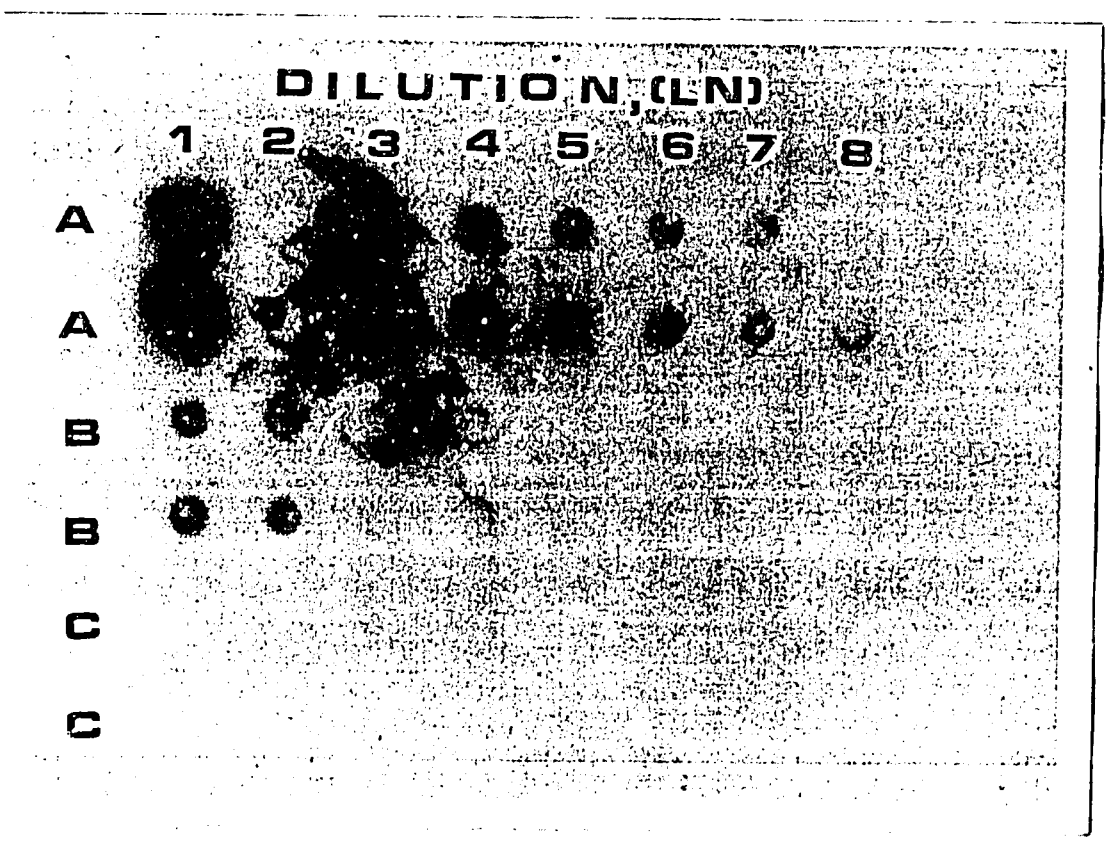

Fig 2. Identification of the extended 3' terminus of the HN cDNA. ssDNA was rescued from the KS(-)47 recombinant by helper phage and sequenced as described in Materials and Methods. The gel shows the plasmid-insert junction. A, C, G, T represent dAMP, dCMP, dGMP and dTMP of the DNA, respectively. The bases indicate the 3' terminus of the HN cDNA (they immediately follow the EcoR1 GAATTC insertion site of the plasmid). The dotted line separates the 20 base terminal sequence.

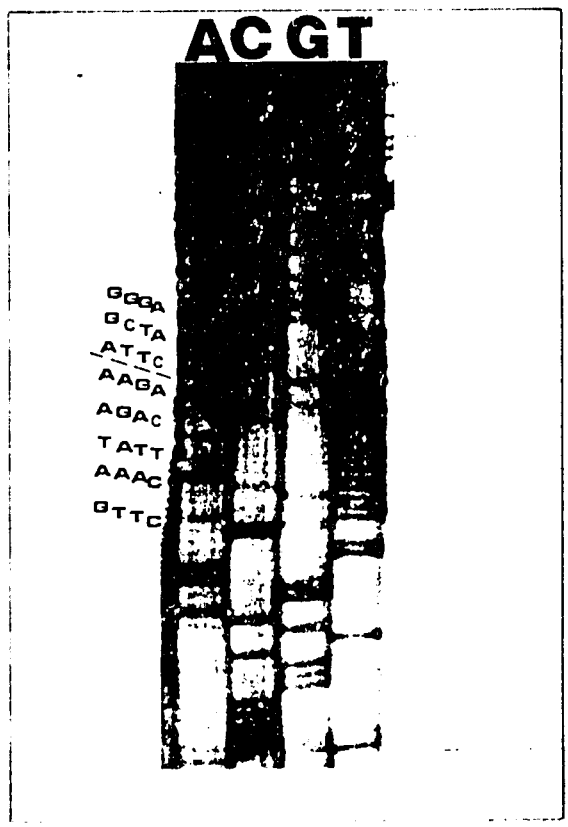

Fig. 3. Comparison of the sequence from Fig. 2 to that of Beaudette C.
A. The complement of the sequence of Fig. 2 that immediately follows the EcoR1 insertion site (lower row) is compared to the HN 3' terminus of Beaudette C (upper row, Millar et al., J. gen. Virol., 1986, 67: 1917).
B. The same sequence (lower row) is compared to the extended HN-L junction of the Beaudette C (upper row, Millar et al., J. gen. Virol., 1986, 67: 1917) in which the sequence that does not belong to the HN is shown in small letters.

A.

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                                        HN gene
5'--->3'                                3' terminus
         NDV, Beaudette C               ----
-CATGCGATCAGATCAAGTCTTGTCAATAGTCCCTCGATTAAG
 :::::::::::::::: :::::::::::::::::::::::::
-CATGCGATCAGATCAATTCTTGTCAATAGTCCCTCGATTAAGTTCTTCTGATAATTTGCAAG
         NDV, Australia-Victoria                                ----
                                                              HN gene,
                                                              3' terminus
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B.

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                                        HN gene        L gene,
5'--->3'                                3' terminus    5' terminus
         NDV, Beaudette C               ----------     ----------
-CATGCGATCAGATCAAGTCTTGTCAATAGTCCCTCGATTAAGAAAAAAtg-taagtggcaat
 :::::::::::::::: :::::::::::::::::::::::::      :: ::: : ::::
-CATGCGATCAGATCAATTCTTGTCAATAGTCCCTCGATTAAGTTCTTCTGATAATTTGCAAG
         NDV, Australia-Victoria                                ----
                                                              HN gene,
                                                              3' terminus
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REFERENCES

1. Millar, N.S., Chambers, P., and Emmerson, P.T., 1986. Nucleotide sequence analysis of the hemagglutinin-neuraminidase gene of Newcastle disease virus. J. Gen. Virol., 67: 1917-1927.

2. Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74: 5463-5467.