PN-ABC-867

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 7/2/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose VO

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. C5-091
PR 10/1/87 - 5/30/88

Attachment

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C5-091

Progress report (June 1, 1988 - December 31, 1988) on the grant # 5544-G-SS-7006-00 "Cloning and Expression of Genes of Newcastle Disease Virus"

Principal Investigators: V. Zaslavsky, MD, PhD
M. Lipkind, MD, PhD

The previous report covered experiments describing the construction of a small library of recombinants (with a plasmid as a vector) as well as the tentative identification of one of them to be NDV HN-specific. The purpose of the construction was to synthesize a cDNA probe for identifying a full-length HN cDNA in an extended library.

This report covers experiments on constructing an extended (master) library (with a lambda gt10 phage as a vector), identification of one of the recombinants as carrying a full (or nearly full) length HN cDNA and attempts to reclone it in a plasmid (for developing a cDNA diagnostic probe as well as for sequencing and expression experiments).

RESULTS

1. Identification of an HN-carrying recombinant plasmid.

i. About 900 base-long insert (marked pND-24) which is HN-specific according to two independent criteria, hybrid selection and restriction mapping (see the previous report), was analyzed further by sequencing its ends. About 160 5'-terminal bases of each strand were sequenced individually in order to identify the precise position of the fragment within the HN gene. Fig.1 compares the end sequences of the insert with the entire HN cDNA, strain Beaudette C (Millar et al, 1986). The positions 590 through 749 are shown as they were read from the gel while the positions 1370 trough 1530 are shown as the complement of the opposite strand. Only few mismatches are seen in Fig. 1. Two conclusions were drawn from this experiment. First, this result confirms the conclusion of the previous report that the pND-24 recombinant carries the HN-specific sequence. Second, the insert (to be utilized for probing the master library) is 941 base long and corresponds to the positions 590 through 1530 of the HN gene

ii. The 941 bp insert was excised with BamH1 from the recombinant plasmid, purified in an agarose gel (preparative scale) and recovered from the gel by electroelution. This preparation was used as a cDNA probe for screening the master library (see below).

3. Construction of master library.

Selected 2-4 kbp fraction of cDNA (see the previous report) was inserted into the EcoR1 site of lambda gt10 as follows:

i. cDNA was methylated, linkered by EcoR1 linkers and ligated to EcoR1-digested phage DNA.

ii. The resulting recombinant DNA's were packaged and used for infecting E. coli containing a high frequency lysogeny (hfl+) mutation (strain NM514). Titration of the progeny showed that about 40,000 phages were expected to be recombinants (by comparison with control). In order to isolate desired recombinants, the progeny was amplified

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and probed with the nick translated HN-specific sequence characterized above.

3. Screening about 40,000 clones from the amplified library revealed about 300 positive (HN-specific) clones.

4. Of these positive clones 43 were analyzed by EcoR1 digestion. Fig. 2 shows one such experiment. It can be seen that a full (or nearly full) length 2 kbp HN cDNA as can be judged from sizes of the EcoR1 fragments (1.3-1.4 and 0.5-0.6 kbp) is present in one of the recombinants (designated lambda b-27) . In addition, a clone containing the same 0.5-0.6 kbp and a larger (1.8 kbp) fragments were also found. It was suggested that the latter clone (designated lambda b-52) contains fused F-HN sequences. This is because NDV-infected cells contain polycistronic mRNA's in addition to individual mRNA's (Wilde and Morrison, 1984), and it cannot be excluded that a part of the upstream (in terms of negative sense NDV genome) F gene in such mRNA was reversely transcribed during cDNA synthesis. If this interpretation is correct (and this will be checked later by both restriction mapping and sequencing), the F-specific sequence will be isolated from the insert, recloned into a plasmid and utilized as a F-specific cDNA probe for fishing out recombinants with full length F cDNA inserts.

3. <u>Subcloning the full-length HN cDNA</u>.

The insert in the lambda b-27 recombinant appears to be the full length HN cDNA according to sizes of 4 individual EcoR1 fragments (about 80-100, 150-200, 450-500 and 1300 bp) found in different digests. This is in good agreement with locations of the 3 EcoR1 internal sites in the HN sequence (Fig. 1). However, since expression of both nonfragmented as well as specifically fragmented full length HN cDNA is the backbone of the entire project, it is crucial to confirm the presence of the entire reading frame in the cloned cDNA. Sequencing the insert, or at least its ends, is the simplest way to achieve that, especially because this provides us also with the information necessary for further expression of cloned cDNA into a polypeptide.

Evident advantages of the Bluescript vectors for subcloning, sequencing and expression make them the prime choice for subcloning the HN cDNA. Excision of the intact insert from lambda-cDNA recombinant, however, encounters a substantial technical difficulty: it is obstructed by presence of 3 internal EcoR1 recognition sites in the insert itself. The following strategy has been employed to overcome this obstacle:

i. generation of the full-length fragment by <u>incomplete</u> EcoR1 digestion of the lambda b-27;
ii. excision of the band corresponding to the "full-length" cDNA;
iii. ligation of the excised DNA to the linearised (by EcoR1) and dephosphorylated plasmid;
iv. transformation of an appropriate E. coli strain;
v. Identification of recombinant plasmids;

The strategy, if successfull, will allow us to be able to excise the full length insert by double BamH1-HindIII digestion (recognition sequences for these enzyme are not present in the HN cDNA but flank

the EcoR1 site in the polylinker region of the bluescripts)

It should be underlined here that only a poor yield of the purified insert is anticipated during incomplete digestion of the recombinant. This is because the size of the insert is small (2kbp) as compared to the size of the phage (more than 40 kbp) and because multiple EcoR1 recognition sites (5) are present in the recombinant (3 internal - within the cDNA and 2 external - at its ends).

Fig 3, A shows one of typical experiments on partial EcoR1 digestion of lambda b-27 DNA. It can be seen that in addition to 1.3 kbp (probably corresponding to the positions 1 through 1299, see Fig. 1) and 0.5 kbp (probably corresponding to the positions 1300 through 1754, see Fig. 1) fragments shown in Fig. 2, two more bands are seen in the partial digest. One of the bands (# 3) is about 600-650 bp long (probably corresponding to the positions 1300 through 1914 with an EcoR1 site at the position 1754 (Fig. 1). The second band (# 1) of about 2 kbp probably represents a mixture of the "full-length" cDNA with a 1.9 kbp fragment (corresponding to the positions 1 through 1914). All the bands were excised separately, eluted and end-labeled with the Klenow fragment of DNA polymerase 1. The result is shown in Fig. 3, B. It is seen in Fig. 3, B that other than 2 kbp fragments are also present in the "2 kbp" fraction. Though the preparation was not homogeneous, it was nevertheless utilized for a shotgun experiment according to the strategy outlined above. We expected to be able to identify the desired recombinant among the clones, but succeeded only in cloning the fragments.

About 500 ug of the purified lambda b-27 DNA was taken for partial digestion in the next experiment. Preparative gels were overloaded with partial digests, horizontal strips of agarose corresponding to the 2 kbp area (according to the marker DNA) were excised from each gel, the eluted DNA's were combined, concentrated and separated by a second round of preparative electrophoresis. This time the 2 kbp material (band #2) was well separated even from the putative 1.9 kbp cDNA (band #3). DNA from band #2 was eluted and concentrated. Though the amount of the material in the final preparation was no more than 10 ng according to ethidium bromide staining, it will be eluted and recloned. Unsuccessful recloning cannot be excluded also here due to unexpectedly low yields of the "full-length" cDNA. Therefore propagation of milligram amounts of the b-27 recombinant has been started in parallel.

The immediate aim of the next experiments (after the 2 kbp fragment is inserted into a Bluescript vector) will be sequencing its ends and an attempt to express it into a polypeptide.

References

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

2. Wilde, A., T. Morrison. 1984. Structural and functional characterization of NDV polycistronic RNA species. J. Virol., 51: 71.

LEGENDS TO THE FIGURES

Fig. 1. Precise location of the cDNA probe within the HN NDV gene.

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 ALIGNMENT OF:
pND-24, strands A (161 b) and B (160 b)
   VERSUS
HN full length (2002 BASES) sequence (Millar et al., 1986)

X         10         20         30         40         50
ACGGGTAGAACGGTAAGAGAGGCCGCCCCTCAATTGGCAGCCGGGCTTCACAACCTCCGT

          70         80         90        100        110
TCTACCGCTTCACCGACAGCAGTCCTCAGTCATGGACCGCGCAGTTAGCCAAGTTGCGTT

         130        140        150        160        170
AGAGAATGATGAAAGAGAGGCAAAAAATACATGGCGCTTGATATTCCGGATTGCAATCTT

         190        200        210        220        230
ACTCTTAACAGTAGTGACCTTAGCTACATCTGTAGCCTCCCTTGTATATAGCATGGGGGC

         250        260        270        280        290
TAGCACACCTAGCGACCTTGTAGGCATACCGACCAGCATTTCTAGGGCAGAAGAAAAGAT

         310        320        330        340        350
TACATCTGCACTTGGTTCCAATCAAGATGTAGTAGATAGGATATATAAGCAAGTGGCCCT

         370        380        390        400        410
TGAGTCTCCGTTGGCATTGTTAAACACTGAGACCACAATTATGAACGCAATAACATCTCT

         430        440        450        460        470
CTCTTATCAGATTAATGGAGCTGCGAACAACAGCGGGTGGGGGGCACCTATCCATGACCC

         490        500        510        520        530
AGATTTTATCGGGGGGATAGGCAAAGAACTCATTGTAGATGATGCTAGTGATGTCACATC

         550        560        570        580        590
ATTCTATCCCTCTGCATTTCAAGAACATCTGAATTTTATCCCGGCGCCTACTACAGGATC
                                                  ::::::::::
                                                  ACTACAGGATC
                                                  X         10

         610        620        630        640        650
AGGTTGCACTCGGATACCTTCATTTGACATGAGTGCTACCCATTACTGCTACACTCATAA
::::::::::: ::::  :::::::::::::::::::::::::::::::::: :::::
AGGTTGCACTCGAATACACTCATTTGACATGAGTGCTACCCATTACTGCTACACCCATAA
        20         30         40         50         60         70

         670        680        690        700        710
TGTAATATTGTCTGGATGCAGAGATCACTCACACTCACATCAGTATTTAGCACTTGGTGT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGTAATATTGTCTGGATGCAGAGATCACTCACACTCACATCAGTATTTAGCACTTGGTGT
        80         90        100        110        120
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          730        740        750        760        770
GCTCCGGACAACTGCAACAGGGAGGATATTCTTTTCTACTCTGCGTTCCATCAGTCTGGA
:::::::::: :::::::::::::: :::
GCTCCGGACATCTGCAACAGGGAGGGTAT ---------------->
         140        150        X

          790        800        810        820        830
TGACACCCAAAATCGGAAGTCTTGCAGTGTGAGTGCAACTCCCTTAGGTTGTGATATGCT

          850        860        870        880        890
GTGCTCGAAAGTCACGGAGACAGAGGAAGAAGATTATAACTCAGCTGTCCCTACGCTGAT

          910        920        930        940        950
GGCACATGGGAGGTTAGGGTTCGACGGCCAATACCACGAAAAGGACCTAGACGTCACAAC

          970        980        990        1000       1010
ATTATTTGAGCACTGGGTGGCCAACTACCCAGGAGTAGGGGGTGGATCTTTTATTGACGG

          1030       1040       1050       1060       1070
CCGCGTATGGTTCTCAGTCTACGGAGGGCTGAAACCCAATTCACCCAGTGACACTGTACA

          1090       1100       1110       1120       1130
GGAAGGGAAATATGTAATATACAAGCGATACAATGACACATGCCCAGATGAGCAAGACTA

          1150       1160       1170       1180       1190
CCAGATCCGAATGGCCAAGTCTTCGTATAAGCCCGGGCCGTTTGGTGGGAAACGCATACA

          1210       1220       1230       1240       1250
GCAGGCTATCTTATCTATCAAGGTGTCAACATCTTTGGGCGAAGACCCAGTACTGACTGT

          1270       1280       1290       1300       1310
ACCGCCCAACACAGTCACACTCATGGGGGCCGAAGGCAGAATTCTCACAGTAGGGACATC
                                          ------
                                          EcoR1
          1330       1340       1350       1360       1370
TCATTTCTTGTATCAGCGAGGGTCATCATACTTCTCTCCCGCGTTATTATATCCTATG-AC
                                                     ::::: ::: ::
                                        <------------ TATCC-ATGTAC
                                                     X160
          1390       1400       1410       1420       1430
AGTCAGCAACAAAACAGCCACTCTTCATAGTCCCTATACATTCAATGCCTTCACTCGGCC
::::::: ::: :::::::::::::::::::: :::::::::::::::::::::::::::
AGTCAGCGACACAACAGCCACTCTTCATAGTCCTTATACATTCAATGCCTTCACTCGGCC
150        140        130        120        110        100

          1450       1460       1470       1480       1490
AGGTAGTATCCCTTGCCAGGCTTCAGCAAGATGCCCCAACTCGTGTGTTACTGGAGTCTA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AGGTAGTATCCCTTGCCAGGCTTCAGCAAGATGCCCCAACTCGTGTGTTACTGGAGTCTA
90         80         70         60         50         40

          1510       1520       1530       1540       1550
TACAGATCCATATCCCCTAATCTTCTATAGGAACCACACCTTGCGAGGGGTATTCGGGAC
::::::::::::::::::::::::::::::
TACAGATCCATATCCCCTAATCTTCTATAG
30         20         10         X
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          1570      1580      1590      1600      1610
AATGCTTGATAGTGAACAAGCAAGACTTAATCCTACGTCTGCAGTATTCGATAGCACATC

          1630      1640      1650      1660      1670
CCGCAGTCGCATAACTCGAGTGAGTTCAAGCAGCACCAAAGCAGCATACACAACATCAAC

          1690      1700      1710      1720      1730
TTGTTTTAAAGTTGTCAAGACCAATAAGACCTATTGTCTCAGCATTGCTGAAATATCTAA

          1750      1760      1770      1780      1790
TACTCTCTTCGGAGAATTCAGAATCGTCCCGTTACTAGTTGAGATCCTCAAAAATGATGG
              ------
              EcoR1
          1810      1820      1830      1840      1850
GGTTAGAGAAGCCAGGTCTGGTTAGTTGAGTCAACTATGAAAGAGCTGGGAAGATGGCAT

          1870      1880      1890      1900      1910
TGTATCACCTATCTTCCGCGACACCAAGAATCAAACTGAATGCCGGTGCGAGCTCGAATT
                                                     -----
                                                     EcoR1
          1930      1940      1950      1960      1970
CCATGTCGCCAGTTGACCACAATCAGCCAGTGCTCATGCGATCAGATCAAGTCTTGTCAA
-
          1990        X
TAGTCCCTCGATTAAGAAAAAA
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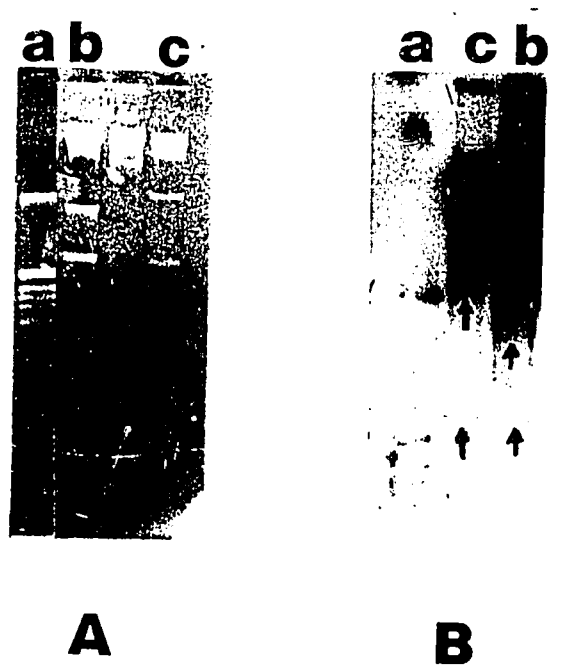
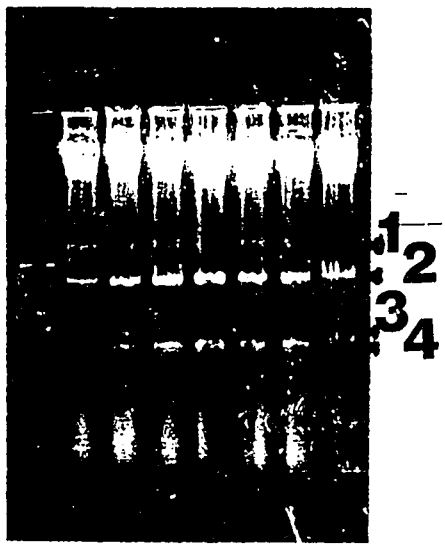
The HN-pBR322 recombinant (pND-24) was purified in a CsCl gradient. 15 ug of the preparation was digested with BamH1 followed by separation of the excised insert (about 0.9 kbp) from the vector by electrophoresis in 1% agarose gel. The insert was isolated from agarose by electroelution, ligated to BamH1-linearized and dephosphorylated Bluescript KS(+) and used for transformation of competent E. coli cells. Single strands of the insert were rescued by a helper M13 phage and sequenced by modified dideoxy method with Sequenase (Stratagene) according to the manufacturer's protocol. The two sequences have been aligned versus full HN sequence, strain Beaudette C (Millar et al., 1986). The upper sequence - full HN sequence. The lower sequences (positions 590 through 749 and 1370 through 1530) - end sequences of the insert. EcoR1 recognition sites are marked along the upper sequence. NIH sequencing package (the January 1988 release) was used for all the computations.

Fig. 2. EcoR1 digestion of lambda gt10 phage recombinants.



Phages that hybridize to the HN cDNA probe were propagated. Extracted DNA's were digested with EcoR1 and either analyzed in a 1% agarose ge directly (A) or after end labeling with the large fragment of DNA polymerase 1 and 32P-dATP (B). a - marker DNA's of 1631, 515, 396, 344, 298 and 220 bp long; b - b-27 DNA; c - b-52 DNA.

Fig. 3. Kinetics of EcoR1 digestion of b-27.



A

B

A. Lambda b-27 DNA (3.5 ug per assay) was digested by EcoR1 for 15, 30, 45, 60, 70, 80 and 90 min and analyzed in a 1% agarose gel. A marker DNA as in Fig. 2 is shown in the left slot (only 1631 bp fragment is visible).

B. End-labeling of individual DNA's. Bands #1 (2kbp), #2 (1.3 kbp), #3 (0.7 kbp) and 4 (0.5 kbp) were cut off separately from the above gel (Fig. 3, A), eluted and aliquotes of each were individually labeled by the Klenow fragment of DNA polymerase 1 and 32P-dATP. a - marker DNA (as in Fif. 2); b - fraction 1; c - fraction 2; d - fraction 3; e - fraction 4.

Progress report (October 1, 1987 - May 30, 1988) on the grant # 5544-G-SS-7006-00 "Cloning and Expression of Genes of Newcastle Disease Virus"

C5-091

Principal Investigators: V. Zaslavsky, MD, PhD

M. Lipkind, MD, PhD

The first 8 month of the research are covered by Step 1 of the research proposal. This step included the following 3 groups of experiments:

i. Synthesis of cDNA's on poly(A)-containing RNA's extracted from NDV-infected cells;

ii. Cloning the synthesized cDNA's;

iii. Identification of clones with the HN specificity;

This has been accomplished. The methodology (originally suggested in the 1985 version of the proposal) was modified where and when it was found necessary to utilize new techniques and developments in the field. This will be described in the text.

RESULTS

RNA isolation.

i. Kinetics of virus-induced RNA synthesis were determined on an analytical scale in small (30 mm) Petri dishes and are shown in Fig. 1. The result demonstrates that synthesis of virus-specific RNA's has been practically completed by 12-16 hours after infection. This time was therefore considered as the optimal one for RNA isolation from infected cells.

ii. Preparative isolation of poly(A)-containing RNA's. Infection of cells was done in 30 large (140 mm) Petri dishes, as described in the legend to Fig. 1. One dish was used for labeling virus-specific RNA's (to produce an internal marker) while the remaining infected monolayers were not labeled. 14 hours after infection the cells were lysed, as described by Maniatis et al. (1982). The NDV RNA-containing ribonucleoprotein fraction was isolated from cytoplasmic extracts as

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described earlier (Zaslavsky et al., 1971.), the RNA's were released from this material by proteinase K digestion (Maniatis et al., 1982) and fractionated in SDS-containing sucrose gradients. RNA's of the 18S zone (marked by 3H-radioactivity) were collected, and the poly(A)-containing fraction was separated from the bulk RNA's by chromatography through an oligo(dT) column. This preparation was utilized for cDNA synthesis.

Synthesis of cDNA. Method of Gubler and Hoffman (1983) was used for cDNA synthesis instead of originally proposed method of Land et al. (1981). The principle of the chosen method is to synthesize the second cDNA strand by generating multiple specific primers rather than by tailing 3' ends of the 1st strand DNA product, as proposed by Land et al.(1981). Though both methods utilize the primed 2nd strand synthesis approaches, Gubler and Hoffman (1983) have eliminated the necessity of tailing the 1st strand cDNA and escaped thus the presence of undesirable non-gene sequences in the final product.

Cloning of cDNA. The cDNA synthesized as described above was ligated to phosphorylated Bam H1 linkers and inserted into the Bam H1 site of pBR 322. E. coli HB101 cells were transformed by the recombinant molecules, and the Amp-resistant, Tet-sensitive colonies were isolated. A library of NDV-carrying inserts was established by blotting the isolated colonies and probing the blots with 32P-labeled viral RNA. The probe was prepared by mild alkaline hydrolysis of viral RNA (extracted from highly purified virus) and kinasing it using 32P(gamma)-ATP and T4 polynucleotide kinase.

Identification of an HN-carrying recombinant plasmid. 5 virus-specific clones from the library were analyzed by hybrid selection test, and one of them has revealed the HN specificity according to this test (Fig. 2). This was confirmed by constructing a limited restriction map of the insert and comparing it with the computed (NIH sequencing package, the December 1987 release) restriction map of the entire NDV HN sequence.

The insert was mapped with pst1, pvuII, EcoRI, HindII, HindIII, and Sal1 (Fig. 3). Fig. 4 shows where in the HN gene the isolated insert is embedded. Neither computed sequences within the domain of interest, nor the isolated insert contained pst1, HindIII, or Sal1 restriction sites. Likewise, no other domain along the entire HN gene contained the restriction sites in the order (PvuII--HindII--EcoR1) found in the positions 601-1401 (Fig. 4). Thus, the insert seems to be HN-specific according to two independent criteria, hybrid selection and restriction mapping. However, the insert is too short (about 800 bp) to be utilized for the purpose of the research. Since the constructed NDV-specific library consists of limited number of clones, it was decided to construct another library using a more efficient cloning vector rather than to look for a larger insert in the library. It is planned to utilize the isolated pBR322-HN recombinant for identification of clones with the HN-specificity in the second library constructed.

As an alternative cloning vector, the lambda gt10 phage had been chosen due to its significantly higher (2 to 3 orders of magnitude) cloning efficiency. In addition, it was decided to clone a fraction of cDNA's with enriched content of molecules of desired size rather than the entire cDNA product. Therefore, cDNA's synthesized as above were fractionated in a sucrose gradient prior to cloning. An aliquot of each fraction of the gradient was analyzed in an agarose gel for measuring the product sizes. Fractions containing 2-4 kbp long molecules were combined, and will be utilized for constructing a library. The next immediate experiments will be concerned with cloning the selected cDNA's (already sitting in the freezer) into the unique EcoR1 restriction site of the lambda gt10 phage, identification of HN-carrying recombinants (by probing the phages with the HN-pBR 322 recombinant described above) and size analysis of the HN-specific inserts.

V. Zaslavsky, MD, PhD

References

1. Gubler, U., Hoffman, B.J., 1983. A simple and very efficient method for generating cDNA libraries. Gene, 27:263.

2. Land, H., Grez, M., Hauser, H., Lindenmaier, W., Schutz, G., 1981. 5'-terminal sequences of eucaryotic mRNA can be cloned with high efficiency. Nucl. Acids Res., 1984, 9., 2251:2266.

3. Maniatis, T.,Fritsch, E.F., Sambrook, J.,1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory.

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

5. Zaslavsky, V., Zaides, V.M., Volkova, M.Y., Kaverin, N.V., Bukrinskaya, A.G., 1971. Virus-specific informosome components in extracts of NDV-infected cells. FEBS Lett.,14:133.

LEGENDS TO THE FIGURES

Fig. 1. Synthesis of NDV-specific RNA's in NDV-infected chicken fibroblast cells

Newcastle disease virus (NDV), strain Australia-Victoria, was plaque-purified and propagated in chick embryos in a conventional manner. Clarified allantoic fluids were used for infecting chicken cell monolayers (30 mm Petri dishes) at multiplicities of infection 10-20. Actinomycin D (2 ug/ml) and 3H-uridine (300 Ci/mmol, 25 uCi/ml) were added to the infected cells 4 and 4.5 hours after infection, respectively. At appropriate times the medium was discarded, monolayers were dissolved in 1% SDS, precipitated with trichloroacetic acid (TCA), and the radioactivities incorporated were measured in a liquid scintillation spectrometer.

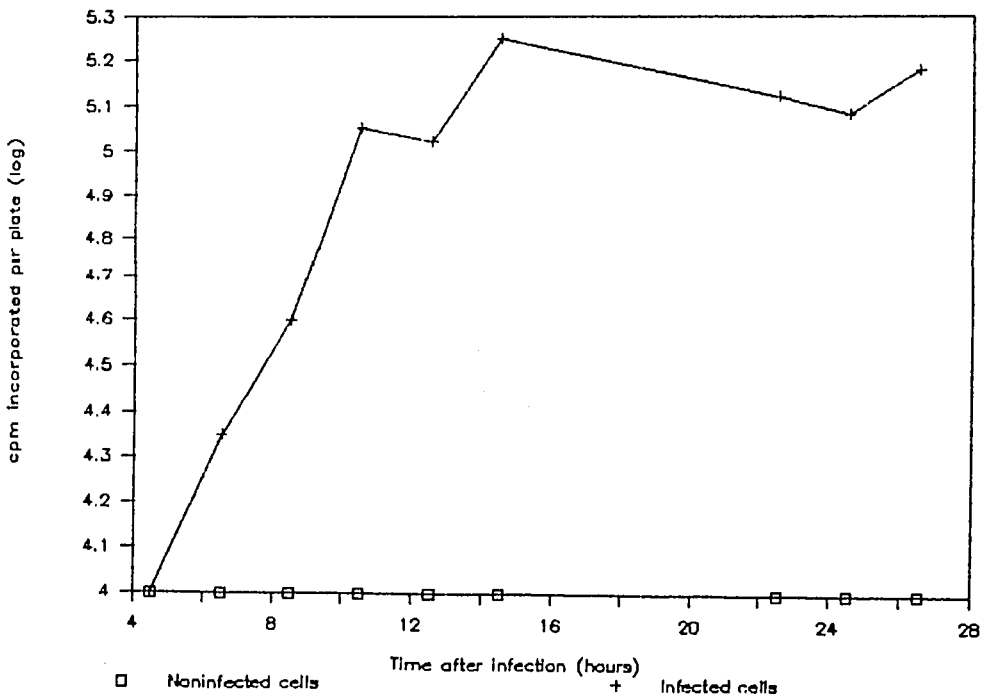
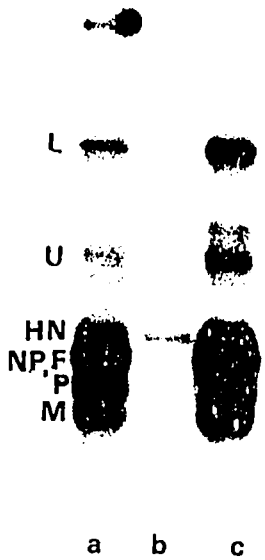


Fig. 2. Gene specificity assigned to pBR322-NDV recombinants in a hybrid selection test.



Chicken cells monolayers have been infected and treated, as in the legend to Fig. 1, except that RNA's were labeled with 32P rather than with 3H. Total RNA was extracted from the cells and hybridized to the purified pBR322-recombinants. Hybrid-selected 32P-RNA's were analyzed in a 1.2% formaldehyde-agarose gel along with the RNA's used for hybridization. The gels were exposed to an X-ray film (intensifying screen, -70 C). a,c- total 32P-RNA; b, - hybrid selected 32P-RNA;

Fig. 3.Restriction mapping of the insert excised from an HN-pBR322 recombinant.

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BamH1     EcoRI     HindII                PvuII      BamH1
    -------|--------|------------------|---------
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The HN-pBR322 recombinant has been purified in a CsCl gradient. 15 ug of the preparation was digested with BamH1 followed by separation of the excised insert (about 0.8 kbp) from the vector by electrophoresis in 1% agarose gel. The insert was isolated from agarose by electroelution and restricted both with single EcoRI, HindII, HindIII, pst1, Sal1, and PvuII endonucleases and with different combinations of EcoRI, HindII and PvuII . The digests were analyzed by electrophoresis in agarose gels. No sites for HindIII, pst1 and Sal1 were found. Locations of EcoRI, HindII and PvuII sites have been identified along the insert as shown.

Fig. 4. Identification of the insert sequences along the HN NDV gene.

```
601   ATTCTTTTCT ACTCTGCGTT CCATCAGTCT GGATGACACC CAAAATCGGA

651   AGTCTTGCAG TGTGAGTGCA ACTCCCTTAG GTTGTGATAT GCTGTGCTCG
                                          PvuII
701   AAAGTCACGG AGACAGAGGA AGAAGATTAT AACTCAGCTG TCCCTACGCT
                                          ------
751   GATGGCACAT GGGAGGTTAG GGTTCGACGG CCAATACCAC GAAAAGGACC

801   TAGACGTCAC AACATTATTT GAGGACTGGG TGGCCAACTA CCCAGGAGTA

851   GGGGGTGGAT CTTTTATTGA CGGCCGCGTA TGGTTCTCAG TCTACGGAGG

901   GCTGAAACCC AATTCACCCA GTGACACTGT ACAGGAAGGG AAATATGTAA

951   TATATAAGCG ATACAATGAC ACATGCCCAG ATGAGCAAGA CTACCAGATC

1001  CGAATGGCCA AGTCTTCGTA TAAGCCCGGG CGGTTTGGTG GGAAACGCAT
                                 HindII
1051  ACAGCAGGCT ATCTTATCTA TCAAGGTGTC AACATCTTTG GGCGAAGACC
                                 -------
1101  CAGTACTGAC TGTACCGCCC AACACAGTCA CACTCATGGG GGCCGAAGGC
       EcoR1
1151  AGAATTCTCA CAGTAGGGAC ATCTCATTTC TTGTATCAGC GAGGGTCATC
       ------
1201  ATACTTCTCT CCCGCGTTAT TATATCCTAT GACAGTCAGC AACAAAACAG

1251  CCACTCTTCA TAGTCCCTAT ACATTCAATG CCTTCACTCG GCCAGGTAGT

1301  ATCCCTTGCC AGGCTTCAGC AAGATGCCCC AACTCGTGTG TTACTGGAGT

1351  CTATACAGAT CCATATCCCC TAATCTTCTA TAGGAACCAC ACCTTGCGAG

1401  GGGTATTCGG GACAATGCTT GATAGTGAAC AAGCAAGACT TAATCCTACG
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The above sequences have been determined from overlapping clones (Millar et al., 1986). The HN gene sequence is shown as that of positive sense viral RNA (5'---3'). The restriction sites found in the insert (see Fig. 2) have been aligned along the above sequence by computation. Only part of the HN gene (positions 601 through 1401) where the insert is embedded is shown for simplicity. No other domain along the entire HN gene contains restriction sites similar to the positions 601-1401.

Nucleotide position	Restriction nuclease	Sequence recognition	Fragment	Size (bp)
737	PVUII	CAG¦CTG		
1080	HINDII	GTY¦PAC	HindII-PvuII	343
1152	ECORI	G¦AATTC	EcoRI-HindII	72

ATTACHMENT 1

PROJECT NO. DPE-5544-G-SS-7006-00
ACTUAL DISBURSEMENTS
FOR THE PERIOD OCT 1, 1987 TO MAR 31, 1988 (FIRST YEAR)
(IN DOLLARS)

BUDGET LINE ITEM	BUDGET	DISBURSEMENTS THIS PERIOD*	CUMULATIVE DISBURSEMENTS	BALANCE
SALARIES	22,000	3,325	3,325	25,325
EQUIPMENT/MATERIALS	28,000	17,419	17,419	10,581
TRAINING/TRAVEL	4,000	4,000	4,000	-
OTHER COSTS	500	-	-	500
OVERHEAD	4,400	665	665	3,735
TOTAL	58,900	25,409	25,409	33,491

The undersigned hereby certifies: A: that payment of the sum claimed is proper and due and that appropriate refund to AID will be made promptly upon request in the event of disallowance of costs not reimbursable under the terms of the agreement; B: that information on the fiscal report is correct and such detailed supporting information as AID may reasonably require will be furnished promptly to AID on request; C: that all requirements called for under the agreement to date of this certification have been met.

BY R. NAVON

TITLE MANAGER, FINANCIAL SECTION

DATE 24.3.88

MINISTRY OF AGRICULTURE
P.O.B. 12, BET-DAGAN

Oct. 1, 1987-Mar. 31, 1988

ATTACHEMENT 2.

Report on visit of Cooperative Investigator, Dr. Marcia Marin (Costa Rica) to Israel (Grant # DPE 5544-G-SS-7006-00)

The purpose of Dr. Marin's visit was of twofold:

1. To get familiar with veterinary virology approaches to be used at a later step in Costa Rica under supervision of Dr. Y. Weisman;

2. To get familiar with molecular biology approaches utilized in the frame work of the research (cDNA synthesis and cloning, clone analysis);

These have been completed during Dr. Marin's one month visit to our lab.

1. Veterinary Virology.

i. Isolation of NDV from poultry;
ii. Antigenic identification of the NDV isolates (HI, ELISA, AGP);
iii. Serological surveillance of poultry for NDV antibodies;
iv. Visiting poultry farms: studying disease control and management;

2. Molecular Biology.

Since cDNA has been already sysnthesized and cloned by the time when Dr. Marin arrived, her training included the clone analysis. Dr Marin participated in screening the clone library for identification of virus-specific clones and restriction analysis of isolated clones. Specifically, Dr. Marin was introduced to the following techniques:

i. Propagation of Newcastle disease virus and its purification in an isopycnic sucrose gradient.
ii. Extraction of viral RNA from the virus;
iii. Preparing a 32P-labeled probe by kinasing the RNA;
iv. Colony hybridization to the probe;
v. Propagation of plasmids;
vi. Isolation of plasmids both at analytical (mini) and preparative scales. The latter included purification of plasmids in CsCl gradients;
vii. Restriction of plasmids with restriction endonucleases;
viii. Analysis of restricted plasmids in agarose gels;