PN-ABL-
75738 C5-091

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FINAL REPORT

Covering period: October 1986 through December 1991

Submitted to the Office of the Science Advisor
U.S. Agency for International Development

Cloning and Expression of Genes of Newcastle Disease Virus

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Project # C5-091
Grant # 5544-G-SS-7006-00

A.I.D. Grant Project Officer: Dr. Charles A. Lawson, Science Attache,
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Project Duration: August 1986 through December 1991

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Executive Summary:

Since synthesis and cloning the full-length HN cDNA is the precondition for successful execution of the project, an approach that secures increased yields of non-degraded RNA templates for cDNA synthesis has been developed first. This has been achieved due to the improvement based on an earlier finding (Zaslavsky et al. 1971) that NDV-induced mRNA's are present in cytoplasmic extracts of infected cells in the form of RNP's, i. e. particles, rather than free RNA's. The developed approach significantly increases the suitability of RNA templates for cDNA synthesis and should be of interest for those who plan to express or otherwise utilize cloned full-length cDNA's.

Sequencing the synthesized HN cDNA has shown that its direct expression could not be done: the upstream HN gene noncoding domain contains a termination TAA codon that precedes the coding domain and is in frame with it. This has been corrected by PCR-created deletion of the entire undesired domain. Both full-length HN cDNA and two of its domains have been successfully expressed into the full HN polypeptide and its fragments, respectively - according to the planned strategy. All the three polypeptide products have been purified for antibody production.

A protocol for utilizing the synthesized and cloned NDV cDNA in the capacity of a probe for NDV identification both in allantoic fluids and clinical specimen has been developed and tested with a very promising result. The synthesized cDNA can be used according to the protocol for NDV diagnostics either as such or after slight modification. The technology has been transferred to Dr. M. Marin, cooperative investigator (Costa Rica).

Complete ORF for full-length HN polypeptide that has been cloned in an expression vector opens a possibility of constructing a live divaccine against NDV and either Marek or fowlpox diseases by inserting the HN cDNA into an appropriate avian viral vector (herpes turkey or fowlpox virus).

Research Objectives:

The research is concerned with three aspects of molecular biology of NDV, specifically with the NDV HN gene. They are: developing a cDNA probe (for early NDV diagnostics), synthesizing a full-length HN cDNA (for future development of a synthetic divaccine by inserting the NDV HN cDNA into an avian viral vector) and testing the hypothesis that distinct antigenic epitopes found in the HN protein are represented by noninterrupted nucleotide sequences in the HN gene. The first two aspects are of obvious importance for agriculture. The third one would add to our knowledge on structural and functional organization of the gene that controls initial stages of infection - the aspect that remains almost untouched in the literature available. A strategy that allows us to address this question has been developed and executed in the framework of this project. It consists of fragmenting the full-length cDNA into distinct and characterized sequences, their individual expression and probing the whole virus for HA and neuraminidase activities with antibodies to the expressed polypeptides. No mapping of the activities along the gene has been done so far with the exception for one neutralizing epitope (Chambers et al., 1988).

Since all the three goals are concerned with the HN gene, an approach to secure full-length HN cDNA synthesis has been developed. The developed approach significantly increases the suitability of RNA templates for cDNA synthesis and should be of interest for those who plan to express or otherwise utilize cloned full-length cDNA's.

Methods and Results:

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

Computer analysis. Aligning two sequences was computed by the Lipman and Wilbur NUCALN (for nucleic acids) or PROTALN (for proteins) algorithms, other computations (restriction analysis, identification of ORF's, translating nucleic acid into polypeptides, etc. - by Mount and Conrad sequencing program DM5 (ver. 5.06). All the programs used are the part of the NIH sequencing package, NIH MBUG, ver. 87.1).

RNA isolation. Preparative extraction of RNA from NDV-infected cells has been preceded by kinetic studies in order to identify optimal time for the extraction. 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. For preparative isolation, two-day old monolayers in 30 large (140 mm) Petri dishes were infected, as described in the legend to Fig. 1. Four hours after infection actinomycin D (2 ug/ml) was added to one plate, and 30 min. later ^{3}H-Uridine (300 Ci/mmol, 25 uCi/ml) was added to the same plate. The remaining plates have been left untreated. The reason of the treatment was to create an internal marker for virus-induced RNA 's synthesized in the cells. 15 hours after infection both treated and non-treated cells were detached from the plates by a rubber policeman, and homogenized. The NDV RNA-containing ribonucleoprotein fraction was isolated from cytoplasmic extracts by centrifugation as described earlier (Caslavsky et al., 1971.), the RNA's were released from this material by proteinase K digestion (Maniatis et al., 1982) and the poly(A)-containing fraction was separated from the bulk RNA's by

affinity chromatography through an oligo(dT) column. Presence of virus-induced RNA's was monitored at all the steps by ^{3}H-radioactivity. The final 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 thus preventing the presence of non-gene sequences in the final product. The cDNA's synthesized were fractionated in a sucrose gradient prior to cloning. An aliquot of each fraction was analyzed in an agarose gel for measuring the product sizes. Fractions containing 2-4 kbp long molecules were combined, the cDNA's were methylated (to protect internal EcoRI sequences from digestion at a later step), ligated to phosphorylated EcoRI linkers and the master library of about 50,000 clones has been established by cloning linkered cDNA's into the unique EcoR1 restriction site of the lambda gt10 phage.

Cloning the cDNA's synthesized in the lambda g10 phage vector. Lambda gt10 phage had been chosen as a cloning vector due to its high cloning efficiency. The cloning has been performed by utilizing the Amersham cDNA cloning kit according to the manufacturer's manual. The master library has been screened with a HN-specific cDNA probe (see below) for clones with the HN specificity.

Preparation of a HN-specific cDNA probe. For identification of recombinant phages carrying desired sequences a specific cDNA probe has been synthesized in a separate experiment. The cDNA was synthesized by the method of Gubler and Hoffman (1983), as above. The product was ligated to phosphorylated Bam H1 linkers and inserted into the Bam H1-linearized pBR322. Competent E. coli cells have been transformed by recombinant plasmids, and Tet-sensitive, Amp-resistant colonies were isolated. A library of virus-specific recombinants was established by blotting isolated colonies and probing the blots with ^{32}P-labeled viral RNA. The RNA probe was prepared by mild alkaline hydrolysis of viral RNA (extracted from highly purified virus) and kinasing it with ^{32}P(gamma)-ATP and T4 polynucleotide kinase. DNA's from 5 virus-specific clones have been extracted and analyzed by hybrid selection test. One clone revealed the HN specificity according to this test (Fig. 2). The HN-specificity of this clone was further confirmed by limited restriction mapping: the insert was excised from recombinant DNA by BamH1 and restricted with Pst1, PvuII, EcoRI, HindII, HindIII, and Sal1 (not shown) of which PvuII, HindIII and SalI restriction sites have not been found in the insert, as expected. Thus, the insert seemed to be HN-specific according to the two independent criteria, hybrid selection and restriction mapping. In order to identify precise location of the cloned domain, about 160 bases have been sequenced from each end of the insert (Fig. 3). The Figure shows that the cloned cDNA is 941 base long and corresponds to the positions 590 through 1530 of the NDV HN gene.

Identification of phage recombinants with the HN specificity. About 300 clones have been identified as HN-specific by screening the master library with the probe characterized above. HN-specific recombinants (9 out of 300) were further purified by the second round of plaque purification followed by hybridization to the same probe, propagated, restricted by EcoRI, end-labeled with large fragment (Klenow) of DNA polymerase I and analyzed for sizes in agarose gels. Fig. 4 shows two such clones. One of them (track "C") contains an insert which is very close in size to the full-length HN gene (about 2 kbp).

This was concluded from presence of 1.4 kbp and 0.6 kbp long fragments in the EcoR1 digest. The other (track "B") - contains the same 0.6 kbp long fragment, but its larger fragment is about 2 kbp long rather than 1.4 kbp. This suggests that the latter cDNA has been synthesized by reverse transcription of a di- or polycistronic NDV mRNA (Wilde and Morrison, 1984) in which case the extra 600-700 bases should belong to the F gene (which is located immediately upstream from the HN gene in the NDV genome). This suggestion has been supported by restriction analysis of the insert (not shown).

Construction of plasmid recombinants carrying full-length HN cDNA. Excision of the intact insert from lambda-cDNA recombinant encountered substantial technical difficulty due to the presence of internal EcoR1 recognition sites in the insert itself. The selected phage (Fig. 4, C) has been propagated, its DNA was purified and digested with EcoRI with the purpose to isolate full-length HN cDNA molecules. Fig. 5 shows the result of this experiment. Presence of the same 1.4 and 0.6 kbp bands as above (Fig. 4, C), and, in addition, 2 kbp- and 0.7 kbp-long bands that are products of incomplete digestion are seen in both panel A and B. Thus, this experiment revealed presence of the second internal EcoR1 site within the 700 bp terminal fragment which was overlooked in the Fig. 4 due to small size of the third band (100 bp). In summary, EcoR1 digestion of the insert generates 3 fragments of 1.4, 0.6 and 0.1 kbp which is in close correlation with calculated 1327, 617 and 86 bp fragments due to presence of the EcoRI sites in the positions 1327 and 1944 of the HN gene (Millar et al., 1986). It was concluded that a full (or nearly full) size NDV HN cDNA is present in the clone analyzed. The 2 kbp fragment (Fig. 5, B, b) has been ligated to the linearized (by EcoRI) and dephosphorylated (by calf intestine alkaline phosphatase) Bluescript KS plasmid. A number of colonies was isolated and those with largest inserts were utilized for further experiments. Fig. 6 shows restriction of a selected recominant. This recombinant (marked KS52) is about 5 kbp long (slot C), contains a 2 kbp insert that can be excised by BamH1-HindIII double digestion (slot D), Pst1 and EcoR1 sites (slots E and F, respectively) positions of which correspond to the reference HN sequence (Millar et al., 1986). The recombinant was sequenced in both directions in order to identify the ends of the cloned cDNA sequence.

Sequencing DNA's. Either purified dsDNA's or rescued ssDNA's were utilized for sequencing by chain termination method of Sanger et al. (1977). In the latter case individual DNA strands were rescued from plasmid (Bluescript) recombinants by M13 helper phage, strain R408 (UBS), according to the manufacturer's protocol. The sequencing assays were electrophoresed in 8% polyacrylamide gels containing 9M urea and exposed to X-ray films for autoradiography.

Precise location of cloned HN cDNA in the KS-HN recombinant. The critical 5' end sequence of the HN cDNA is shown in Fig. 7. Aligning the sequence along the theoretical construct containing the HN (NDV, strain Beaudette C) sequence (Fig. 8) shows that the synthesized cDNA lacks only 7 terminal bases (ACGGGTA) from its very 5' end and hence is practically full-length equivalent of the gene (panel A). Panel B shows fully preserved 3' end of the gene, as expected. Occasional mismatches are probably because the compared HN sequences belong to different NDV strains: strain Australia-Victoria in our study and strain Beaudette C used for aligning as a reference. This construct has been used as such for developing a NDV-specific cDNA probe.

Developing a cDNA diagnostic probe. The KS52 recombinant has been utilized for probing NDV, and the result is shown in Fig. 9. The protocol includes virus disintegration by SDS, blotting the disintegrated virus to a nitrocellulose

membrane (without prior RNA extraction), and probing the membranes with ^{32}P-labeled recombinant DNA. DNA labeling has been done by random priming method that in our hands resulted in 5- to 10-fold higher specific activity of probes as compared to the nick-translation method. Further improvement can be achieved if the cDNA insert is cut off from the recombinant DNA (by double BamHI-HindIII digestion. Successful utilization of a clone containing the HN-specific sequence for probing the NDV genomes is demonstrated in Fig. 9 where blotted NDV (rows A and B), but not influenza virus (row C) reveal clear hybridization signals. Three conclusions can be made from the Figure:

i. the developed protocol permits blotting of viral RNA without its prior extraction, no matter whether highly purified virus (A) or crude preparation (B) is to be probed.

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

iii. Sensitivity of the method is not affected by a very large excess of contaminating proteins that are present in allantoic fluid: initial titer of the allantoic virus was 100 times lower as compared to the purified preparation (which corresponds to the 5 ln difference shown in the figure).

Thus, the cloned NDV-specific cDNA is suitable for specific recognition of NDV. Applicability of this result for diagnostics requires, however, additional experiments on identification of the virus in clinical specimen directly. This has been done by probing swabs from infected birds. It was found that insoluble impurities that are present in swabs cause rather significant non-specific hybridization signals, and therefore prior relative purification is unavoidable here. Two more steps have been included into the protocol. First, treatment of swabs with a non-ionic detergent (in order to release viral cores) followed by low speed centrifugation for separating heavy particles. Second, high speed centrifugation of the supernatants for pelleting viral cores. The final pellets (not always visible) were solubilized in small volumes (20 ul) of 1% SDS, and blotted, as above. Infected chicken (1, 3 and 5 days after infection, three birds in each group) have been tested. Two (out of three) swabs (3 day after infection) and all the 3 swabs (5 days after infection) revealed clear positive hybridization signals whereas testing swabs from 1 day-infected birds was negative. It is unclear whether presence of sufficient number of viral genomes in swabs depends on infection stage (which would explain the result with 1 day infected birds), or further improvement of the approach is necessary. The latter could be done by inserting additional NDV sequences into the recombinant (these sequences can be easily fished out from the master library that has been constructed during execution of the project).

Construction of an expression recombinant. Isolation of a clone that contains the entire HN open reading frame (ORF) is an evident condition for successful research, and this condition has been fulfilled (Fig. 8). However, the HN cDNA insert cannot be expressed as such in the above construct for two reasons. First, both vector-coded GAL and HN sequences must be in the same ORF (which they are not) in order to produce a fused GAL-HN polypeptide. Second, the upstream non-coding HN gene domain contains the initiation codon (ATG) that is preceded by an in-frame located termination codon (Fig. 8). Therefore expression of the HN cDNA requires its prior modification which has been done by PCR-created deletion of the HN upstream non-coding domain.

Modification of the HN cDNA insert by PCR. PCR assay has been set according to a described protocol (Dangler et al., 1990) for cyclic amplification of DNA sequences. Two primers have been synthesized for amplification of the desired sequence. Fig. 10, A shows that the synthesized 32 base-long start primer

contains a part of the plasmid's polylinker region (pos. 2241 through 2254) that is immediately followed by the HN coding sequence (pos. 2344 through 2361). The primer lacks the vector's EcoRI site (GAATTC) but contains the CCATGG NcoI restriction sequence instead (the sequence will be unique in the construct). This arrangement has been utilized later for both identification purposes and constructing an independent expression recombinant (see below). In summary, it was expected that the entire upstream noncoding domain (85 bases, positions 2259 through 2343) will be deleted, and both genes will share the same ORF (Fig. 10, D). The end primer (Fig. 10, B) was a complement to a sequence that is located about 950 bp downstream the gene and contains the AatII and NheI unique restriction sites. The PCR product is shown in Fig. 11, A, track 4. The synthesized fragment is of expected size: a single 850 bp band is present in the track 4, and this band is about 100 bp shorter than the 950 bp HindIII-AatII fragment excised from the KS52 (track A).

Construction of the expression recombinant. The PCR product shown in Fig. 11, A (track 4) has been double-digested with HindIII and NheI (a unique restriction sequence which is not present in the plasmid part of the recombinant). The resulting 161 bp fragment was ligated to the purified 4727 bp fragment of the HindIII-NheI double-digested parental KS52 recombinant (Fig. 11, B), and competent cells were transformed with the ligation assay. The desired recombinant (marked KS-HN) has been identified by presence of newly created unique NcoI site, and both cDNA junctions have been sequenced. This is shown in Fig. 12. Two conclusions can be drawn from the Figure, where these sequences (lower rows) are plotted against the expected ones (upper rows). First, the coding sequence of HN cDNA insert immediately follows the vector's sequence, i.e. the entire noncoding region has been deleted from the 5' terminus of the cDNA (panel A). Second, the 3' end of the insert is identical to that of the previously sequenced KS52 (panel B). Since the excised fragment is of 1.9 kbp in length, we have concluded that the full-length HN cDNA (except for the deleted upstream non-coding region) is present in the recombinant. HN cDNA sequence in this recombinant is in frame with that of the vector's betagalactosidase gene, as expected (Fig. 10, D).

Expression of the construct. Cells were propagated until late log phase (0.5-0.7 optical density at 600nm) and induced with IPTG for 2 hours. The cells were lysed with SDS, and lysates were analyzed in SDS-containing 10% polyacrylamide gels. The analysis revealed presence of a clear 65-70 K band (according to a protein molecular weight markers), which was not present in both lysates of KS52- and vector-transformed cells (not shown). Thus, the expression construct that consists of 4888 bp (Fig. 13) has been synthesized. Even though construction of the desired recombinant was technically successful, low quantity of the expressed polypeptide has made it necessary to look for a better expressing system. It was decided to make two other expression constructs utilizing vectors with stronger promoters. Of these, one was an expression vector with strong thermoinducible lambda promoter (pRIT2T). This vector has an additional advantage - presence of a protein A gene - which should allow us to be able to purify and concentrate the expressed protein A-HN fused polypeptide by one-step affinity chromatography through immobilized IgG. The second expression vector, high efficiency T7 RNA polymerase vector, pET (Rosenberg et al., 1987) was chosen because it permits selective synthesis of non-fused polypeptides (see below).

Construction of pRIT2T-HN expression recombinant. The precondition for successful construction of a pRIT2T-HN expression recombinant was shifting the ORF for HN in order to have the HN coding sequence in frame with that of the

pRIT2T protein A gene (Fig. 14). This has been achieved by inserting the 8 mer EcoRV linker into unique EcoRV site of the KS-HN (Fig. 13). Linkered constructs (Fig. 14, A) have been identified by the presence of newly created MamI restriction sequence (GATnnnnATC). The pRIT2T-HN recombinant (Fig. 14, B) has been created by inserting the EcoRV-BamHI 1.9 HN cDNA fragment into the SmaI-BamHI digested pRIT2T (both EcoRV and SmaI create blunt, i.e. compatible ends). The construct is to express a polypeptide that consists of protein A to which the entire HN polypeptide (577 amino acids) is fused (Fig. 14, C).

Expression of the pRIT2T-HN construct has been induced by the temperature shift from 30 C to 42 C after transformed cells reached late log phase. The cells were lysed with SDS two hours after induction, and the lysates have been analyzed in SDS-containing 10% polyacrylamide gels. Surprisingly enough, expression of the HN polypeptide by this construct was rather low as well. Chromatography of cell lysates through immobilized IgG did not help - either due to large quantity of contaminating bacterial proteins or presence of the expressed polypeptide in a sedimentable fraction of the lysate rather than in a supernatant.

Construction of the pET8c-HN expression recombinant . Family of pET expression vectors has been developed by Studier and coworkers (Studier and Moffatt, 1987; Rosenberg et al., 1987; Studier, et al., 1990) for high efficiency expression of cloned genes. The vectors contain T7 RNA polymerase promoter under control of which an inserted gene is to be placed for selective high level expression. This is because the promoter is specifically recognized by T7 but not by E. coli RNA polymerase. Thus, selective expression of an inserted gene in any vector of the family depends on presence of T7 RNA polymerase, synthesis of which can be selectively induced by IPTG in an appropriate host.

The pET8c member of the T7 RNA polymerase family expression vectors (Studier and Moffatt, 1987) has a particular advantage for insertion the HN cDNA: the vector contains the upstream NcoI site ccATGg (the ATG of which is in-frame located) and the downstream BamHI site (Fig. 15, a). Since the KS-HN recombinant (Figs. 13 and 15, b) contains these restriction sites flanking the HN cDNA, construction of an expression recombinant is a simple one-step procedure (Fig. 15, c).

Expression of the pET8c-HN construct. Expression of the HN cDNA by this construct has been induced exactly as that of KS-HN recombinant (see above). Fig. 16 shows one of the experiments that demonstrates expression of the HN cDNA by two independently isolated recombinants. It can be seen that a polypeptide of about 63-65K is synthesized in induced cells (tracks 3 and 4, right, large arrows) in contrast to non-induced cells (tracks 3 and 4, left). The polypeptide is not present in cells transformed with the vector alone (tracks 1), no matter whether they are induced (right) or not (left). However, expression of the HN polypeptide is lower than that of an unrelated gene inserted into the same pET8c vector (track 2, right). The latter recombinant was constructed with the purpose to demonstrate extremely high expression potentials of the pET8c expression vector. The reason of weaker expression of the HN cDNA in each of the 3 expression vectors used is unknown, although some speculations could be made. One is that the HN synthesis in NDV-infected cells is accompanied by glycosylation of nascent polypeptides - the event that could stabilize the product. If true, lack of glycosylation in a bacterial host may lead to either impaired stability of the product, or its premature termination. The latter is indirectly supported by the finding that in some experiments shorter polypeptides have also been found (Fig. 16, tracks 3 and 4, small arrows), as if

one of the TGG (Trp) and TGT (Cys) codons has been recognized as the TGA nonsense codon (TER). Sizes of the polypeptides (35K and 50K) correspond to the Trp in the pos. 312 and Cys in the pos. 454 of the HN polypeptide, respectively.

Construction of recombinants expressing 5' domains of the HN cDNA. The strategy that has been elaborated for separate expression of individual gene domains is described in detail below. Briefly, if a sequence containing an in-frame termination codon is introduced into the cDNA insert, its expression will be terminated at the point of insertion. Only desired 5' gene domain (mRNA polarity) will be expressed by such recombinant. Likewise, if the second NcoI restriction site is in-frame inserted into the cDNA, the 5' domain can be excised by NcoI digestion, and the recircularized recombinant will express only the 3' domain of the gene. The construction could be simplified by inserting only one sequence in which the in-frame termination codon is followed by the in-frame NcoI restriction sequence.

In principle, DNA technology allows us to insert any sequence into any desired location - the question is to do that by the simplest, i.e. time- and effort-saving way. In the case of HN cDNA, a unique PvuII site is present approximately in the middle of the 1.9 kbp cDNA, and this has been utilized for inserting the synthesized sequence. However, successful construction of the linkered pET8c-HN recombinant requires prior elimination of the vector's PvuII site that otherwise would interfere with the construction. Therefore the 4693 bp pET8c has been double-digested with PvuII and NruI (both enzymes create blunt ends and are unique in the vector) and recircularized into the 3599 bp pET8c-DEL. The entire HN cDNA was reinserted into this modified vector resulting in a 5504 bp construct (pETHN-DEL) that contains a single PvuII site (Fig. 17, A). Two complementary sequences, 5'-CTTAATGACCCATGGA and 3'- GAATTACTGGGTACCT (an extra "C" that precedes the NcoI site is added in order to have the in-frame ATG) have been synthesized and annealed. Insertion of the linker (Fig. 17, B) resulted in creating a recombinant (marked pETHN-5') that contained two additional TER codons (positions 1275 and 1278) as well as additional NcoI site (pos. 1282). Thus, it was expected that the recombinant would express a polypeptide synthesis of which starts at the position 480 and stops at the position 1275, i.e. 265 amino acid-long protein coded by the 5' domain of the HN gene.

Construction of recombinants expressing 3' domains of the HN cDNA. Expression of the 3' HN cDNA domain (mRNA sense) has been achieved by one step modification of the recombinant described in the previous section. Namely, pETHN-5' recombinant (Fig. 17, B) was digested with NcoI and recircularized (Fig. 17, C). This resulted in excision of 803 bases and formation of a construct (marked pETHN-3') that translates the downstream 942 bases into the 314 amino acid-long polypeptide (cumulative length of the expressed polypeptides is 579 (and not 577) amino acids due to the inserted 6 base NcoI sequence).

Expression of the 3'domain of the HN cDNA. Expression of the pETHN-3' recombinant has been induced by IPTG, as described above. Fig. 18, tracks 2 and 3 show synthesis of the full-length HN polypeptide (see also Fig. 16). Expression of the 3' gene domain by pETHN-3' recombinant is shown in the tracks 7 through 9. The synthesized polypeptide is present in the sedimentable fraction (track 9) and not in the supernatant (track 8).

Preparation of antigens for immunization. Individual HN-specific polypeptides have been prepared by induction of pETHN, pETHN-5' and pETHN-3'recombinants in 100 ml cultures, as described. Cells were pelleted by centrifugation at 8000xg, lysed by 1% SDS and electrophoresed in preparative 12% polyacrylamide gels. The 65K (577 amino acid full-length HN polypeptide), 30K

(265 amino acid polypeptide expressed from the 5' gene domain) and 36K (314 amino acid-long polypeptide expressed from the 3' gene domain) bands have been cut off for immunization of rabbits.

Preparing antibodies against individual HN-specific polypeptides. Antisera to individual antigens are yet to be isolated: less-than-expected expression in all three expression vectors utilized caused substantial delay in collecting sufficient amounts of corresponding proteins (see the "Methods and Results" section). Six rabbits (two per antigen) will be immunized shortly, and this part of the project is in progress.

DISCUSSION

Synthesis of full-length HN gene was the precondition for successful execution of the project: the project is concerned with analysis of the expressed polypeptide product.

The result presented in the report shows that full-length HN gene of NDV (strain Australia-Victoria) has been successfully synthesized and cloned. An approach for improved RNA isolation has been developed with the purpose to enrich RNA preparations with non-degraded RNA templates for cDNA synthesis. The approach is based on an earlier finding (Zaslavsky et al. 1971) that NDV-induced mRNA's are present in cytoplasmic extracts of infected cells in the form of 40 S RNP's, i.e. particles, rather than free RNA's. The finding opens a possibility of separating mRNA's from soluble RNases prior to RNA isolation preventing thus hydrolytic degradation of RNA templates and supposedly securing their integrity for cDNA synthesis. It was demonstrated that 5' mRNA termini are preserved in all the nine randomly chosen HN-specific clones analyzed (out of 300 isolated), i.e. that the clones contain practically complete DNA equivalents of the HN RNA gene (Zaslavsky and Molad, 1990). Sequencing the ends of the cDNA showed that not only the complete ORF for the entire 577 amino acid-long HN polypeptide but also the upstream noncoding domain (except for seven 5'-terminal bases) is present in the cDNA. Moreover, about 600 3'-terminal bases of the F gene (that is located immediately upstream from the HN gene in the NDV genome) was found to be present in two clones (one of them is shown in Fig. 4, B). The finding suggests that even polycistronic NDV mRNA's (Wilde and Morrison, 1984) retain their integrities and can be utilized as templates for cDNA synthesis. Thus, the modified approach significantly increases the suitability of RNA templates for cDNA synthesis and should be of interest for those who plan to express or otherwise utilize cloned full-length cDNA's.

The strategy for mapping antigenic epitopes and functional HN activities is based - according to the proposal - on probing the virus with antibodies to polypeptides expressed from known domains of the gene. Even though such domains have been successfully cloned (Fig. 17) and expressed (Fig. 18), corresponding antisera are yet to be isolated: less-than-expected expression in all three expression vectors utilized caused substantial delay in collecting sufficient amounts of corresponding proteins (see the "Methods and Results" section).

Chambers et al. (1988) reported recently their results on mapping HN epitopes on the HN gene. The strategy used by these authors is different from that employed by us and is based on analysis of monoclonal antibodies by series of overlapping HN polypeptide fragments. Having some advantages, the approach possesses an important disadvantage: each effort-consuming experiment is strictly limited by testing a single monoclonal antibody, i.e. only a single epitope can be mapped in each series of experiments. Since our approach gives a possibility of identifying neutralizing, HA and NA epitopes, it looks promising

to combine both approaches, i.e. to map groups of epitopes first and then - isolate monoclonals to the individual polypeptide fragments and analyze them directly.

Isolation of practically full-length HN cDNA is of special importance for one of the applied aspect, developing a synthetic anti-NDV vaccine which is planned to be done in the framework of an independent project. This project will include insertion of the HN cDNA (isolated and characterized above) into an avian viral vector (herpes turkey or fowlpox viruses that are currently used as vaccines against Marek disease and fowlpox, respectively) with the purpose to develop a divaccine against NDV and one of the above avian diseases. Developing the vaccine will probably require isolation of also the F cDNA which is not at our disposal at present. However, one of the isolated clones contains short (about 600 bp) F-specific sequence fused to the full-length HN cDNA (see p. 7 of the report) which can be utilized as the probe for fishing out the full-length F cDNA from the master library, as was done with the HN cDNA.

Another project objective was identification of NDV genomes in clinical specimen by their direct probing with a NDV-specific cDNA probe. The main advantage of the developed protocol is the possibility of probing viral genomes without their prior extraction from clinical specimen - the procedure that would not only complicate the diagnostics but could even jeopardize it due to substantial losses of RNA in the process of its extraction from samples with low concentration of the virus. It was found that probing the virus is rather sensitive, reproducible and is not affected by presence of proteins in large excess (Fig. 9). The virus can be identified in swabs as early as 3 and 5 days after infection, but not 1 day after infection. It is unclear whether presence of sufficient number of viral genomes in swabs depends on infection stage, or further improvement of the approach is necessary. The latter could be done by inserting additional NDV sequences into the recombinant (these sequences can be easily fished out from the master library that has been constructed during executing the project).

In principle, PCR opens an alternative way for identification of a specific sequence. This method is extremely sensitive and a single DNA molecule in a solution could theoretically be identified by its amplification. Unfortunately, the method cannot be applied to RNA NDV genomes directly: viral RNA must be first extracted and then converted into the cDNA. Recently, however, thermostable DNA polymerase with reverse transcriptase activity became available commercially which means that both reactions - reverse transcription of RNA and cDNA amplification can be performed in the same tube. This increases potential applicability of PCR for NDV diagnostics. The question only remains whether or not viral genomes could be released and amplified without their prior extraction from clinical specimen. As was mentioned above, this is important in two respects. First, chances to isolate RNA from very small amounts (that are expected in the specimen) are rather slim. Second, the necessity to extract RNA substantially complicates the diagnostic routine. It was reported recently (Jestin & Jestin, 1991) that NDV genomes can be identified in allantoic fluids by PCR, but the described approach has little, if any, diagnostic value: the authors used about 200 ul of allantoic virus which is about 1000 times more than is required for cDNA probing (Fig. 9).

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 1-2. Actinomycin D (2 ug/ml) and ^{3}H-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. +, infected monolayers. Open squares, noninfected control.

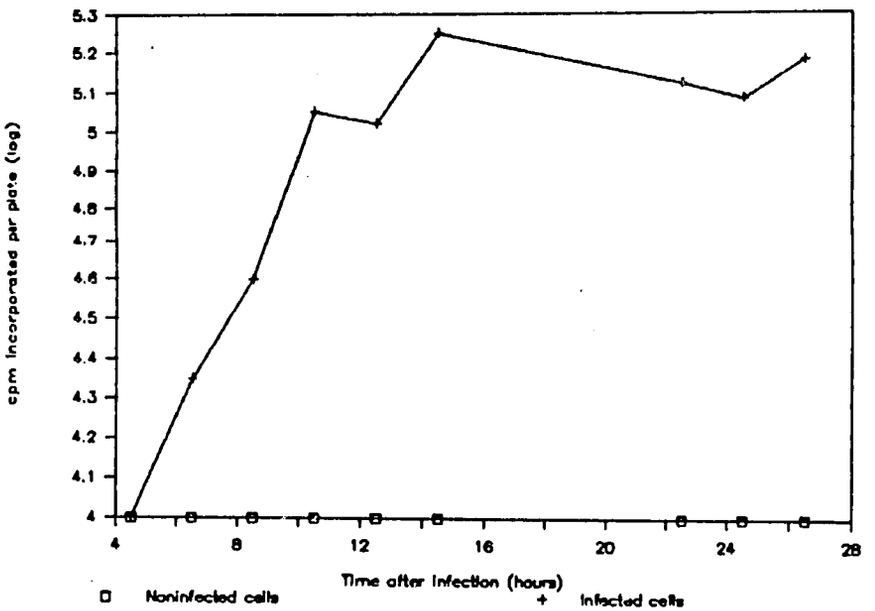


Fig. 2. The HN gene specificity assigned to a pBR322-NDV recombinant in a hybrid selection test. Chicken cells monolayers have been infected and treated, as in the legend to Fig. 1, except that RNA's were labeled with ^{32}P rather than with ^{3}H. Total RNA was extracted from the cells and hybridized to the purified pBR322-recombinants. Hybrid-selected ^{32}P-RNA's (b) were analyzed in a 1.2% formaldehyde-agarose gel along with total ^{32}P-RNA's prior to hybridization (a, c). The gels were exposed to an X-ray film (intensifying screen, -70 C). Positions of individual viral mRNA's are indicated at the left side of the Figure.

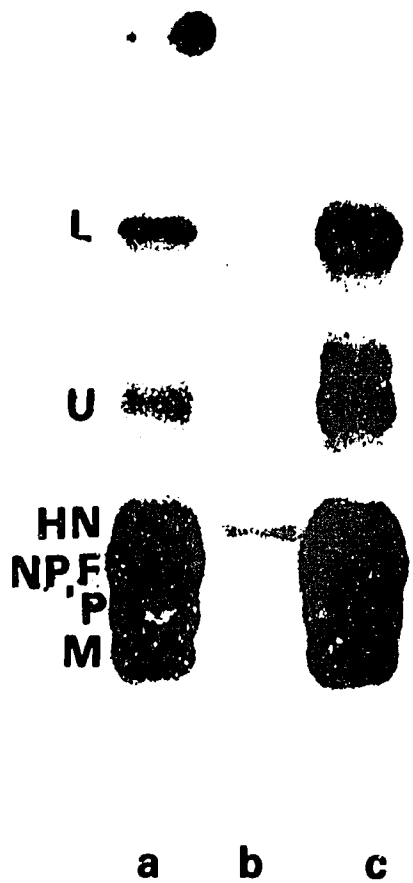


Fig. 3. Precise location of the cDNA probe within the NDV HN gene. 15 ug of CsCl-purified HN-pBR322 recombinant was digested with Bam HI followed by preparative electroelution of the excised 0,9 kb insert from an agarose gel. The insert was ligated to Bam HI-linearized and dephosphorylated Bluescript vector, and competent E. coli cells have been transformed with the ligation assay. The insert has been sequenced in both directions by modified dideoxy method (Sanger et al., 1977) with both universal and reversed M13 primers. The two sequences were aligned versus full HN sequence, strain Beaudette C1 (Millar, et al., 1986) as the reference. The upper sequence - Beaudette C1 HN sequence. The lower sequence - end sequences of the insert (the positions 590 through 749 are shown as they were read from the gel while the positions 1368 through 1530 are shown as the complement of the opposite strand).

```
                                        580       590
                                        CCCGGCGCCTACTACAGGATC
                                                  ::::::::::
                                                  ACTACAGGATC

         610       620       630       640       650
AGGTTGCACTCGGATACCTTCATTTGACATGAGTGCTACCCATTACTGCTACACTCATAA
:::::::::::: ::::  :::::::::::::::::::::::::::::::::: :::::
AGGTTGCACTCGAATACACTCATTTGACATGAGTGCTACCCATTACTGCTACACCCATAA


TGTAATATTGTCTGGATGCAGAGATCACTCACACTCACATCAGTATTTAGCACTTGGTGT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGTAATATTGTCTGGATGCAGAGATCACTCACACTCACATCAGTATTTAGCACTTGGTGT


         730       740       750                 1370
GCTCCGGACAACTGCAACAGGGAGGATATT------------------ATATCCTATG-AC
:::::::::: :::::::::::::: :::                     ::::: ::: ::
GCTCCGGACATCTGCAACAGGGAGGGTAT--------------------TATCC-ATGTAC
                            X                     X

         1390      1400      1410      1420      1430
AGTCAGCAACAAAACAGCCACTCTTCATAGTCCCTATACATTCAATGCCTTCACTCGGCC
::::::: ::: :::::::::::::::::::: :::::::::::::::::::::::::::
AGTCAGCGACACAACAGCCACTCTTCATAGTCCTTATACATTCAATGCCTTCACTCGGCC


         1450      1460      1470      1480      1490
AGGTAGTATCCCTTGCCAGGCTTCAGCAAGATGCCCCAACTCGTGTGTTACTGGAGTCTA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AGGTAGTATCCCTTGCCAGGCTTCAGCAAGATGCCCCAACTCGTGTGTTACTGGAGTCTA

         1510      1520      1530
TACAGATCCATATCCCCTAATCTTCTATAGGAACC
::::::::::::::::::::::::::::::
TACAGATCCATATCCCCTAATCTTCTATAG
```

Fig. 4. EcoRI digestion of HN-specific phage recombinants. Phages that hybridize to the HN-specific cDNA probe were propagated. Extracted DNA's were digested with EcoRI, end-labeled with the large fragment of DNA polymerase 1, analyzed in an 1% agarose gel, and autoradiographed. A, marker DNA's of 1631, 515, 396, 344, 298 and 220 bp long; B and C - two different recombinant DNA's.

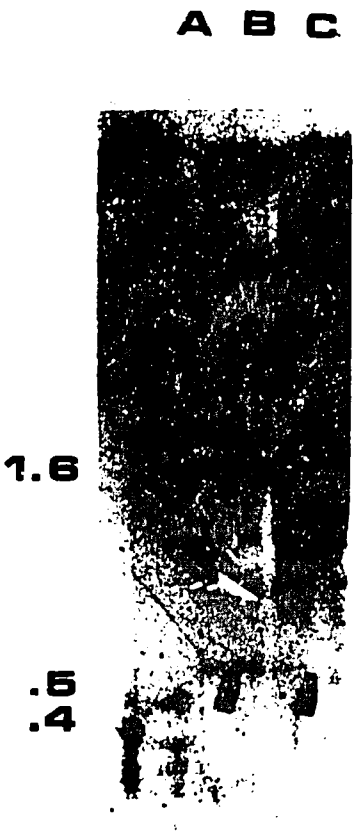


Fig. 5. Incomplete EcoRI digestion of the HN-page recombinant. A: DNA of the page shown in Fig. 4, C (3.5 ug per assay) was digested by EcoRI for 15, 30, 45, 60, 70, 80 and 90 min and analyzed in a 1% agarose gel. Marker DNA, as in Fig. 4, is shown in the left slot. B: End-labeling of individual DNA's. Bands 1 (2 kbp), 2 (1.3 kbp), 3 (o.7 kbp) and 4 (o.6 kbp) were cut off separately from the above gel (Fig. 5, A, 15 min digestion), eluted and aliquotes of each were individually labeled by the Klenow fragment of DNA polymerase I and ^{32}P-dATP. The preparations were electrophoresed in a 1% agarose gel and autoradiographed. a, marker DNA (as in Fig. 4); b, fraction 1; c, fraction 2; d, fraction 3; e, fraction 4.

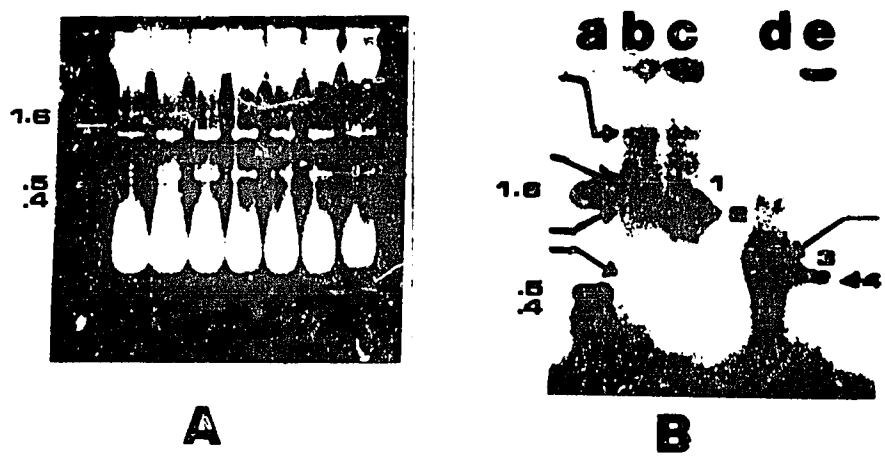


Fig. 6. Restriction analysis and size determination of the KS52 plasmid recombinant. KS52 has been isolated by a modified method (Zaslavsky and Shihmanter, 1988; Molad and Zaslavsky, 1990) from a 1.5 ml overnight culture, restricted and electrophoresed in a 1% agarose gel. A, Bam H1-linearized Bluescript vector (3 kbp). B, HinfI-digested pBR322 (size marker). Slots C through F show restriction of the recombinant KS52 with different restriction enzymes: C, BamH1; D, BamH1 and HindIII double digestion. E, Pst1. E, EcoR1. Size values (in kbp) are shown at the left side of the figure.

Fig. 7 . Sequencing of the 5' terminus of the cloned HN-cDNA. ssDNA was rescued from the KS52 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.

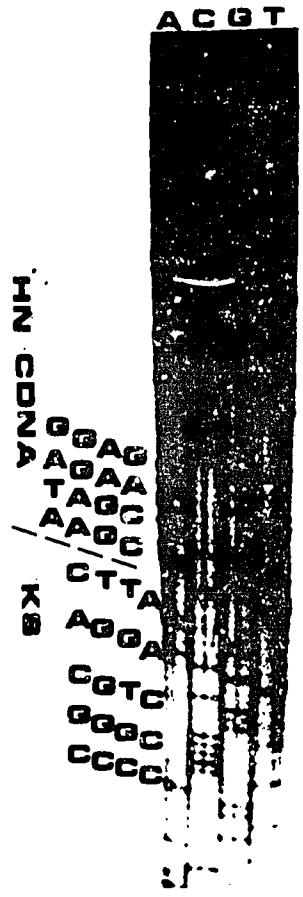


Fig. 8. Identification of both ends of the HN cloned sequence. DNA has been isolated from an overnight culture (10 ml), purified and sequenced (Sanger et al., 1977) with either universal M13 (A) or reversed (B) primers (about 3 ug of the purified preparation per each primer) for identification of the 5' and 3' plasmid-insert junctions, respectively. The upper rows in both panels show reference Beaudette C1 HN sequences (Millar et al., 1986) , the lower - sequences read from appropriate gels. The initiation codon (ATG) of the single open reading frame as well as the in-frame termination codon TAA of the upstream noncoding domain are marked on the sequence. The sequence in panel B is shown as the complement to that of the sequencing gel and plotted against the 3' end of the Beaudette C1 HN sequence (the same strand and the same direction as in the panel A).

A. 5'cDNA junction in the insert-plasmid recombinant.

```
                                                          Beaudette C1
                                                          HN cDNA -->
                                                     5'- ACGGGTAGAA
                                                          |      :::
 ==========tggcggccgctctagaactagtggatcccccgggctgcaggaattc-------gaa
                     plasmid                          ------   cDNA-->
                                                      EcoR1
         20         30         40         50         60
CGGTAAGAGAGGCCGCCCCTCAATTGGCAGCCGGGCTTCACAACCTCCGTTCTACCGCTT
:: :::::::::::::::::::::::  ::: :::::::::::::: :::::::::::
cgaTAAgagaggccgcccctcaattgcgagctgggcttcacaacctctgttctaccgctt
   TER
         80         90         100        110        120
CACCGACAGCAGTCCTCAGTCATGGACCGCGCAGTTAGCCAAGTTGCGTTAGAG--------------
:::::::::::::::::::::::::::::::::::::::::::::: :::::::
caccgacagcagtcctcagtcATGgaccgcgcagttagccaagttgtgttagag--------------
                     MET (Start)---HN--------------------->
```

B. 3'cDNA junction in the insert-plasmid recombinant.

```
     Reference NDV sequence (Beaudette C1)

5'TGCGATCAGATCAATTCTTGTCAATAGTCCCTCGATTAAGAAAAAAAAAAAAAAAAA
  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::: ======
5'tgcgatcagatcaattcttgtcaatagtccctcgattaagaaaaaaaaaaaaaaaaaggaattcct 3'
                  ------>cDNA----------------------------->|EcoRI
                                                            plasmid
```

Fig. 9. Probing NDV with KS52 in the capacity of a diagnostic cDNA probe
Purified ($2x10^4$ HA) and allantoic ($2x10^2$ HA) NDV as well as allantoic influenza virus ($2x10^2$ HA) have been diluted 100 fold and treated (50 C, 1 hr) with equal volumes of PBS containing 2 M glyoxal and 1% SDS. Serial 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 ^{32}P (10^6 cpm/ml of the hybridization mixture). A, purified NDV. B, allantoic NDV. C, allantoic influenza virus;

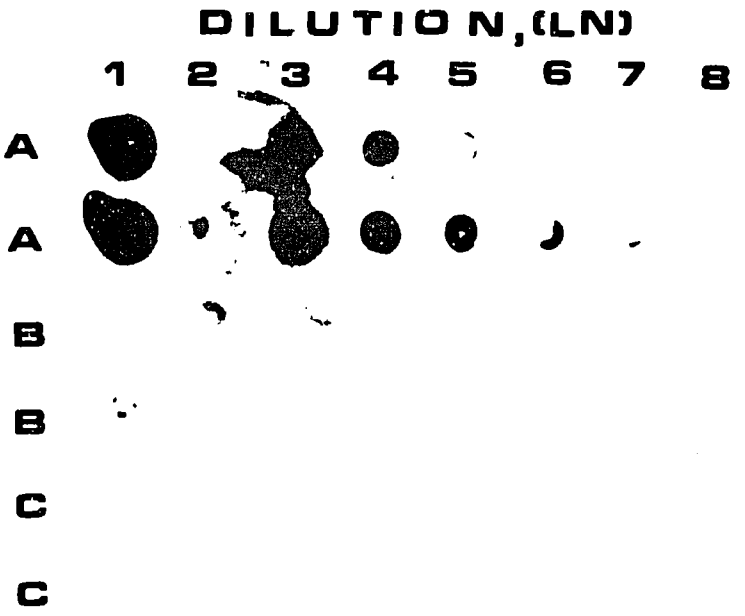


Fig. 10. Modification of the HN cDNA by PCR

A. Start primer (32mer, positions in the KS52: 2241-2254 and 2344-2361).

```
        Bluescript          |--> HN cDNA
                vector      |noncoding domain)  | coding domain-->
          AAGCTTGATATCGAATTCcgaacgg===gtcctcagtcATGGACCGCGCAGTTAG--> 3'
          ::::::::::::    TO BE DELETED        ::::::::::::::::::
        gcaagcttgatatc-------------------------cATGgaccgcgcagttag
     2241         2254                      2344 MET         2361|
        |-------------------START PRIMER-------------------------|
```

i.e. the below sequence should replace the noncoding domain:

```
                                        Nco1
                                        ------
                          5'-gcaagcttgatatccATGgaccgcgcagttag-3'
                                Vector ---->|HN cDNA----->
```

B. End primer. End primer (26 mer complementary to the pos. 3196-3221, 3'-cc tgg atc tgc agt gtt gta ata aac -5') should be utilized for synthesis of the desired 877 base-long fragment (2344 through 3221).

```
      -->HN cDNA-->                         |3196----------------3221|
     5'-----------TTCGACGGCCAATACCACGAAAAGGACCTAGACGTCACAACATTATTTG -3'
                                            |||||||||||||||||||||||||
                                         3'-cctggatctgcagtgttgtaataaac -5'
                                            |                        |
                                            |----END PRIMER----------|
```

C. Final recombinant (KS-HN)

```
    HindIIIEcoRV  MET (HN START)                AatII
      ------====== ---                          ------
5' gcAAGCTTGATATCcATGGACCGCGCAGTTAG---AAAGGACCTAGACGTCACAACATTATTTG 3'
      Bluescript  |------------> HN cDNA (894 bp)
```

D. ORF for the fused polypeptide in the final KS-HN construct

```
5'c cct cga ggt cga cgg tat cga taa gct tga tat ccA TGG ACC GCG CAG->
  Pro Ser Arg Ser Thr Val Ser Ile Ser Leu Ile Ser MET Asp Arg Ala Val
  BETAGALACTOSIDASE---->                              | HN PROTEIN
```

Fig. 11. The PCR-synthesized fragment (A) and purification of HindIII-NheI double-digested KS52 (B). A. The reaction that contained 1 ug of the purified KS52 recombinant, the start and end primers (0.02 ug of each), PCR mix and Taq polymerase, was covered with mineral oil, and 40 amplification cycles were performed. The PCR product was extracted with chloroform and analyzed on an agarose gel. M, molecular weight markers (HinfI-digested pBR 322). A, HindIII-AatII double-digested KS 52. 1 through 3, PCR control assays (different single ingredients are missing). 4, full PCR assay. B. KS 52 (10 ug) has been double-digested with HindIII and NheI and run in an agarose gel for separation of the fragments (tracks A). M, HindIII-linearized KS 52. The arrows show the excised 260 bp fragment to be replaced by the 153 bp HindIII-NheI fragment excised from the material shown in the panel A.

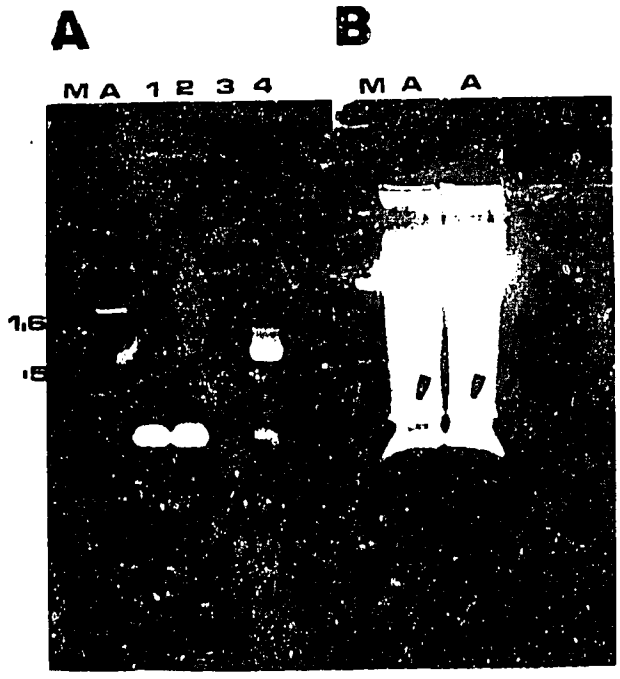


Fig. 12. Identification of both ends of the HN cloned sequence in the PCR-modified KS52 (KS-HN) recombinant DNA has been isolated from an overnight culture (10 ml), purified and sequenced with either universal M13 (A) or reversed (B) primers (about 3 ug of the purified preparation per each primer) for identification of the 5' and 3' plasmid-insert junctions, respectively. The upper rows show expected (theoretical) sequences, the lower - actual sequences.

A. The 5'insert-plasmid junction of the recombinant KS-HN (universal M13 primer)

```
   Polylinker of the vector  HindIII      |HN cDNA----->
5'cccccccctcgaggtcgacggtatcgataagcttgatatccATGGACCGCGCAGTTAG CAAGTTGCGT
  ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
5'cccccccctcgaggtcgacggtatcgataagcttgatatccATGGACCGCGCAGTTAGCCAAGTTGCGT
                                          ======
                                          NcoI
```

B. The 3'insert-plasmid junction of the recombinant KS-HN (reversed M13 primer). The sequence is shown as the complement to that of the sequencing gel and plotted against the 3' end of the same strand and in the same direction as in the panel A.

```
                                                                EcoRI
5'TGCGATCAGATCAATTCTTGTCAATAGTCCCTCGATTAAGAAAAAAAAAAAAAAAAAAgGAATTCctg
  ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
5'TGCGATCAGATCAATTCTTGTCAATAGTCCCTCGATTAAGAAAAAAAAAAAAAAAAAAggaattcctg

                  ------>cDNA------------------------------>|Vector
```

Fig. 13. KS-HN recombinant.

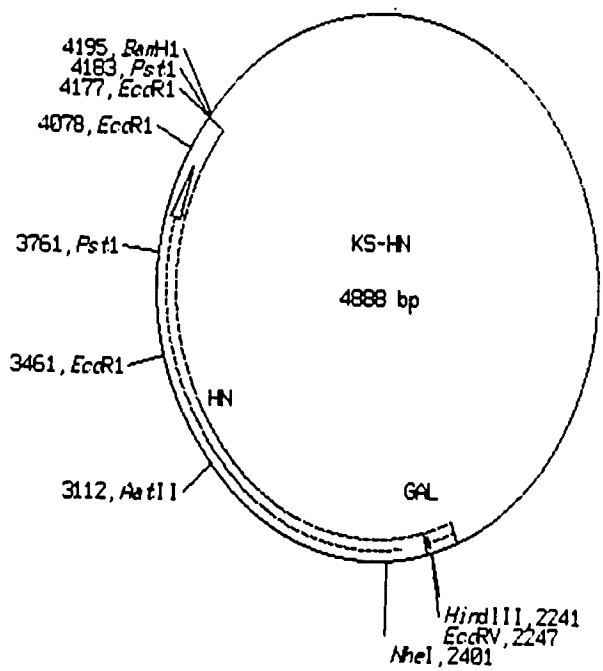


Fig. 14. Construction of pRIT2T-HN expression recombinant.

A. Linkering KS-HN. 8 mer EcoRV linker, GGATATCC, has been inserted into the EcoRV site of the KS-HN (see Fig. 12, A).

```
            HNcDNA---->
 MamI       CcATCcATGGACCG--------G
            T     MET              G
 EcoRV      A                      A  BamHI
            T                      T
            A        <---------    C
            GgTAG_________________C
                  Bluescript KS
```

B. The final construct (pRIT2T-HN)

```
                                HNcDNA
                          ca tcc ATG gac cgc gca gtt agC
                   MamI   c      MET Asp Arg Ala-->    |
                          t                            |
                          a                            |
                          G             <--------      |
                          GC_____________________ CCTAGG
                                   pRIT2T         BamHI
```

C. Expression of the fused protein A-HN polypeptide

```
5'CCG GGG AAT TCC CGG ATC Cca tcc ATG gac cgc gca gtt agC-->
  Pro Gly Asn Ser Arg Ile Pro Ser MET Asp Arg Ala Val Ser-->

   ------ Protein A gene ------>I HN cDNA----->
```

Fig. 15. Construction of pET8c-HN expression recombinant.

a. The pET8c expression recombinant (Rosenberg et al., 1987)

```
      GCTAGCATGACTGGTGGACAGCAAATGGGTCGCg
      G                                 G
      T MET      pET8c                  A
      A                                 T
NcoI  C                                 C BamHI
      C_________________________________C
```

b. The KS-HN (see Fig. 12, A nd B)

```
            HNcDNA----->
            GACCGC------------------------G
            G                             G
      NcoI  T                             A  BamHI
            A  MET    KS-HN               T
            C                             C
            C_____________________________C
                <-------Bluescript KS
```

c. The final construct (pET8c-HN)

```
      GACCGC____________________________g
      G       HN cDNA------->           G
      T MET                             A
      A          pET8c-HN               T
NcoI  C                                 C BamHI
      C_________________________________C
         <---------peT8c
```

Fig. 16. Expression of the pET8c-HN. Cells transformed with the pET8c vector itself (negative control, tracks 1), pet8c containing an unrelated gene (positive control, tracks 2), and two pET8c-HN recombinants (tracks 3 and 4) have been either induced with 1 mM IPTG (right) or noninduced (left). Two hours after induction the cells were collected, lysed and analyzed in an SDS-containing 10% polyacrylamide gel.

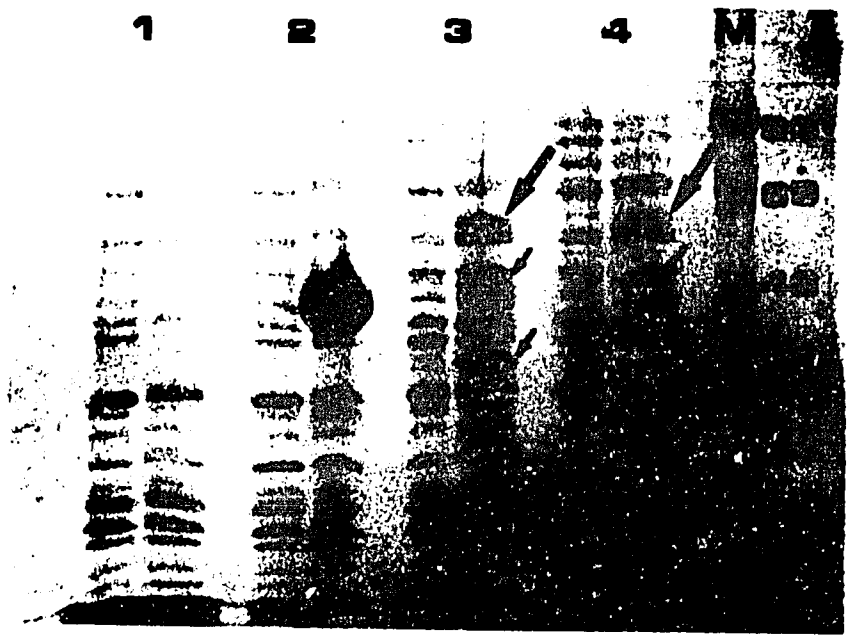


Fig. 17. Construction of pETHN-5'and pETHN-3' recombinants expressing 5' and 3' domains of the HN cDNA.

A. PvuII-digested pETHN-DEL

```
          HN cDNA--->

G--------------ttataactCAG   CTGtccc-----AAAA
G                        PvuII              |
T MET                                       |
A            pETHN-DEL (5504 bp)            |
C                                           |
C___________________________________________|

             <--------vector
```

B. The final construct (pETHN-5')

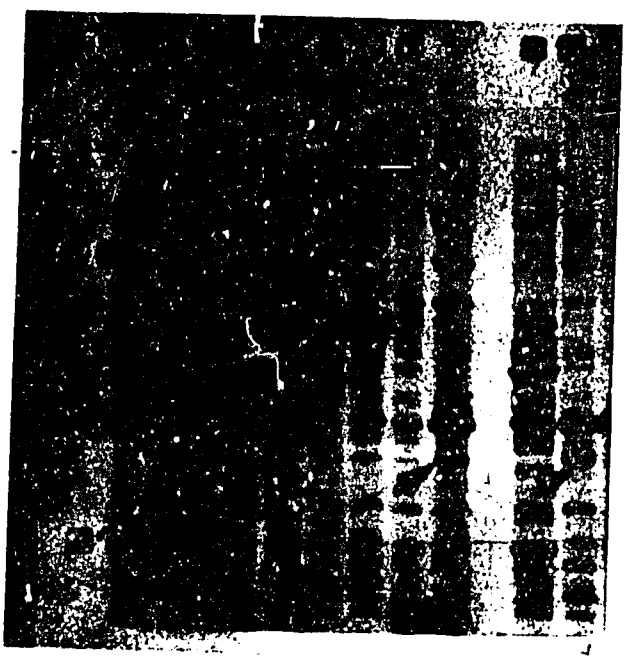
```
HN cDNA----->     TER TER NcoI--->
G----ttataactCAGcttaatgacccatggCTGtccc-----A
G                                          A
T            pETHN-5'                      A
A MET                                      A
C                                          |
C__________________________________________|_
              <--------vector
```

C. The final construct (pETHN-3').

```
  Vector---->
-------------------------------------c
|                                     c
|          pETHN-3'                   a
|                                     t NcoI
|                                     g
|___AAAAA_____________________cctGTCg
             <---HN cDNA
```


29

Fig. 18. Expression of the 3' HN gene domain. Cells, transformed with pET8c-HN (tracks 1 through 3) or pETHN-3' (tracks 6 through 9) were propagated until 0.5 optical density (at 600 nm) at which point IPTG was added (tracks 2 through 5 and 7 through 9) to induce gene expression or not added for control (tracks 1 and 6). After one (track 2 and 4) or two (tracks 3, 5, 7, 8, 9) hours of aerated incubation in presence of the inducer cells were centrifuged, lysed by SDS (tracks 1 through 7) or by freezing-drying (tracks 8-9) and analyzed in a SDS-containing 12% polyacrylamide gel. Tracks 1 through 7 show supernatants after lysing the cells with 1% SDS. Tracks 8 shows the supernatant after freezing-drying cells, whereas the track 9 shows SDS-treated pellet from the same cells. Molecular weights of protein markers (track M) are shown at the left.



Impact, Relevance and Technology Transfer:

A detailed protocol for early NDV diagnostics by cDNA probing has been transferred to Costa Rica. The protocol was tested at an experimental scale with good results, but its field applicability is yet to be evaluated at an outbreak of the infection. If successful, the protocol could replace the existing laboratory diagnostic of NDV that requires prior propagation (in chick embryos) of HA-positive materials for serological NDV identification and takes at least 4-5 days.

Project Activities/Outputs:

The following publications appeared in peer-reviewed journals:

1. Zaslavsky, V., Lipkind, M., and Weisman, Y. 1987. Molecular Biology of RNA Animal Viruses of Agricultural Importance and Applied Aspects in Veterinary Medicine. 2. Paramyxoviruses. Isr. J. Vet. Med., 43: 242-263
2. V. Zaslavsky and T. Molad. 1990. A possible approach to enrich cDNA yields with full-length molecules. Virus Genes, 4:53-62.
3. T. Molad and V. Zaslavsky. 1990. Improved analytical isolation of bacterial plasmids. DNA and Protein Engineering Techniques, 2:103-104.

Project Productivity:

The project accomplished two of the proposed goals. First, an NDV-specific cDNA has been synthesized and cloned. A protocol for utilizing this cDNA in the capacity of a probe for NDV identification in clinical specimen has been developed and tested with a very promising result. Second, complete ORF for full-length HN polypeptide has been synthesized and cloned in an expression vector. The sequence that encodes for the entire 577 amino acid-long HN polypeptide is ready for insertion into an avian viral vector (herpes turkey or fowlpox virus) with the possibility of constructing a live divaccine against NDV and either Marek or fowlpox diseases. The third goal, mapping the HN protein activities along the HN gene, has been accomplished partly. Namely, the cloned full-length HN cDNA has been fragmented into distinct and characterized sequences - according to the proposed strategy. All the sequences have been cloned, individually expressed, and the expressed polypeptides were collected for immunization of rabbits. Due to technical difficulties of expressing the HN cDNA (see progress reports), immunization could not be done in time. It is planned to finish this part in the near future. No mapping the activities along the NDV HN gene has been done so far by others, except for a single neutralizing epitope (Chambers et al., 1988).

Future Work:

1. Produce antibodies to both full HN polypeptide and its fragments that are already expressed in the framework of the project.
Probe the whole virus for HA and neuraminidase activities with these antibodies in order to map the activities along the gene. Depending on the result - analyze the fragment coding for the acivities further employing the same strategy.
2. Isolate from the master library a clone carrying ORF for full-length F cDNA with the purpose to insert it along with the ORF for full-length HN cDNA (isolated and characterized in the framwork of this project) into an avian viral vector. The purpose of this research is to develop a divaccine against NDV and either Marek or fowlpox disease by inserting the NDV sequence into one of these viral genomes. The latter two viruses have been developed into vectors by others and probably are available by cooperation.
3. To test the developed NDV-specific cDNA probe under field conditions.

Literature Cited:

1. Chambers, P., Nesbit, M. Yusoff, K., Millar, N.S., Samson A.C.R., and Emmerson, P.T., 1988. Location of a neutralizing epitope for the hemagglutinin-neuraminidase glycoprotein. J. gen. Virol., 69: 2115.

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

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*Fig. 1. Restriction of analytical preparation of the HN cDNA-plasmid recombinant. The recombinant has been extracted as described in text. **A**: BamH1-linearized Bluescript (3 kbp). **B**: HinfI-digested pBR322 (size marker). Slots **C–F** show restriction of the recombinant with different restriction enzymes: C, BamH1; D, BamH1 and HindIII; E, PstI; F, EcoR1. Size values (in kbp) from the slots A and B are shown at the left side of the figure.*

neously. In addition, multiple transfers make processing rather time-, effort-, and material- (microfuge tubes, pipettor tips, etc.) consuming. Omitting this step and employing a one tube protocol has resulted in easy handling of reasonably large number of samples and speeding up of the processing. In summary, the original boiling method for rapid plasmid isolation [1] was modified and adopted for analytical mini scale preparation. The resulting one-tube protocol saves time, effort, materials, makes it possible to handle a reasonably large number of clones, and secures isolation of 2–4 μg of restriction quality plasmid DNA per tube in 35–40 min. The protocol has been routinely used by us for 2 years with no problems and was found reliable and convenient.

Acknowledgments

Assistance of Mrs. E. Shihmanter is appreciated. The research was supported by AID CDR (USA) grant DPE-5544-G-SS-7006 and the Israel Egg and Poultry Board grant 847-0157.

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DNA and Protein Engineering Techniques

Volume 2, Number 5/6, 1990, Pages 89–108

Improved Analytical Isolation of Bacterial Plasmids

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A boiling method (Holmes and Quigley: *Anal Biochem* 114:193–197, 1981) for rapid isolation of bacterial plasmids has been modified by omitting some steps from the original technique. Preparation of plasmids according to the modified protocol is a simple one tube isolation which is effort-, time-, and material-saving. The protocol permits easy handling of reasonably large number of samples simultaneously. Restriction quality DNA (2–4 μg) is routinely made from 1.5 ml of an overnight culture in no more than 40 minutes.

Analytical ("mini") scale isolation of plasmids is indispensable when a clone of interest is to be identified among other clones in a library. A popular alkaline method [2] requires several hours which is often the reason for delaying restriction of isolated plasmids until the next day. This, in turn, forces an investigator to delay analysis of the next group of clones for 1 more day (in order to prepare the next set of overnight cultures). Thus 2 working days may be required for analysis of each set of clones. Simultaneous processing of a large number of colonies is limited in laboratories with limited manpower and equipment, and efficiencies of such laboratories would be doubled if analysis of each group of clones could be completed within a single day. Moreover, the efficiency of plasmid isolation would be improved further if the number of clones per group could be increased. We have attempted to achieve that by modifying the boiling method [1] of cell lysis. Some steps have been omitted from the original technique, and the protocol was adopted to preparing plasmids in the same tube throughout the entire processing. The final one-tube protocol is simple, permits easy handling of increased number of clones simultaneously, and requires as little as 35–40 min to make restriction quality plasmid DNA sufficient for 4–6 assays.

Generation of expected restriction fragments from a recombinant was considered as the criterion for quality of isolated plasmids and therefore for applicability of the technique for plasmid isolation. A plasmid recombinant with known sequence of its cDNA insert has been utilized to demonstrate the modified approach. Hemagglutinin-neuraminidase (HN) cDNA of Newcastle disease virus (NDV), which is such DNA [3–7], was synthesized and cloned in the EcoR1 site of Bluescript KS(+) plasmid vector (Stratagene). Sequencing the ends of the recombinant (not shown) demonstrated that it corresponds to the positions 8 through 1916 of the reference HN cDNAs [4,5]. A detailed protocol for plasmid isolation is as follows:

1. Spin down 1.5 ml of an overnight culture in a microfuge for 1–2 min, discard supernatant, and resuspend pellet in 1 ml of TNE (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA).

2. Repeat centrifugation, discard supernatant, and resuspend the washed pellet in 100 μl of TNE containing 2 μg/μl lysozyme.

3. Incubate 15–20 min. at room temperature, and place the tube in boiling water for 1 min. Cool rapidly on ice.

4. Centrifuge in a microfuge (with refrigeration) for 10 min and collect the supernatant which is the final preparation.

Figure 1 shows the recombinant treated with restriction enzymes of choice. The linearized recombinant (4.9 kbp) is shown in slot C. Double digestion with BamH1 and HindIII is shown in slot D. These recognition sequences are located at the either side of the EcoR1 cloning site while absent from the HN cDNA. The 3 kbp plasmid and 1.9 kbp insert are seen in the slot. Pst1 digestion is shown in slot E. Since one pst1 recognition sequence is present in the vector's polylinker (close to the 5′ end of the inserted cDNA) and two are in the cDNA at the positions (5′→3′) 210 and 1596 [4], three fragments (3.3, 1.4, and 0.2 kbp) are expected in the assay. Two larger bands are seen in the slot, as expected, while a 0.2 kbp fragment is masked by the presence of low molecular weight material. EcoR1 digestion (slot F) excises the cDNA and cuts it into 1.3 and 0.6 kbp fragments according to the internal EcoR1 recognition sequence at the position 1299 [5].

The results presented demonstrate that DNA sufficient for 4–6 restrictions can be isolated in one microfuge tube promptly and conveniently. The described protocol has an obvious advantage over the original technique according to which a sequential chain of transfers (six–seven) from tube to tube takes place at the phenol extraction step. Obviously, each transfer is accompanied with certain losses and, what is more important, multiple transfers per each individual clone put a limit to number of clones to be processed simulta-

REVIEW

MOLECULAR BIOLOGY OF RNA ANIMAL VIRUSES OF AGRICULTURAL IMPORTANCE AND APPLIED ASPECTS IN VETERINARY MEDICINE. PART 2. PARAMYXOVIRUSES

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1. INTRODUCTION

Paramyxoviruses, like picornaviruses discussed in the previous communication [136] include infectious agents that are important for both medicine and agriculture. One of these agents, Newcastle disease virus (NDV), is an important pathogen for poultry, and countries with developed poultry industries suffer from substantial economical losses due to out breaks of the infection. The family para myxoviridae has been classified [76] int the following genera:

i. Paramyxovirus (paramyxoviru group);
ii. Morbillivirus (measles rinderpest-distemper (MRD) group;
iii. Pneumovirus (respiratory syncytial virus group).

NDV belongs to the paramyxovirus group which also includes 6 types of parainfluenza viruses (types 1 through 5 and turkey/Ontario), parrot paramyxovirus, mumps, and Yucaipa viruses. The members of all three genera, as well as related rhabdoviruses, were studied extensively, since all of them are important pathogens, either human or animal. These viruses are characterized as containing single-stranded negative sense RNA genomes, glycolipoprotein external envelope and inner core, of which the genome is an integral part. Thus these viruses are significantly more complex than the picorna group and organized in an entirely different manner. The peculiarities of their organization secure their functioning, and it seems that this organization is identical throughout all the nonsegmented negative strand viruses. Therefore, though attention mainly will be paid to paramyxoviruses, other nonsegmented negative strand viruses will be also discussed.

2. STRUCTURE OF THE VIRAL GENOME

Paramyxovirus (PMV) genomes are represented by single-stranded RNA molecules which cannot induce infection, if deproteinized: the genomes have their genetic information in a form that is complementary in base sequence to viral messenger RNAs (mRNAs). This was first shown in the mid-sixties [9,56] for NDV and soon after [114] — for vesicular stomatitis virus (VSV). Since mRNA has been defined as positive sense RNA for all the systems [3] the novel kind of genome organization — the negative sense single stranded RNA genome — has been thus established by these publications.

The transcription of paramyxovirus genomes is the first synthetic requirement for promoting the infection: in contrast to positive strand RNA picornaviruses, whose genomes are directly translated into proteins, only complementary copies of paramyxovirus genes are recognized and translated by ribosomes. Therefore the paramyxovirus genomes should be first transcribed in order to satisfy the requirement in mRNAs for protein synthesis. This step is secured by presence in the virion of nucleotide polymerizing enzymes. The identical strategy is employed also by some other related viruses (rhabdo-, orthomyxo-, bunya-, arenaviruses) on which basis all of them are classified as negative strand viruses. Genomes of some viruses that belong to this group, are fragmented: their genes are represented by individual segments of the genome (orthomyxoviruses, arenaviruses). The purpose of the review is to discuss molecular biology of nonsegmented negative strand viruses which is the basis for modern trends in controlling diseases caused by these viruses.

PMVs — like all the negative strand viruses — consist of two structural units: internal ribonucleoprotein core (nucleocapsid) and an outer shell (envelope). Each PMV genome occupies a single nucleocapsid. The proteins of this nucleocapsid secure transcription of the genome (see below) and that is why the isolated nucleocapsides are capable of synthesis *in vitro* of both message and genome RNAs [44,99] or mRNA can be transcribed *in vitro* from the viral

VIRUS GENES 4:1, 63–72, 1990
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A Possible Approach to Enrich cDNA Yields with Full-Length Molecules

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Received October 15, 1989
Revised and accepted January 3, 1990

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Key words: cDNA synthesis, mRNA, NDV, hemagglutinin-neuraminidase gene.

Abstract

Newcastle disease virus (NDV)-specific hemagglutinin-neuraminidase (HN) mRNAs were used as the model templates for cDNA synthesis. Polyadenylated RNAs were isolated from the particulate fraction of cytoplasmic extracts of NDV-infected cells rather than from nonfractionated extracts. The approach is based on earlier findings that eucaryotic mRNAs are present in cytoplasmic extracts in the form of ribonucleoproteins (mRNPs) rather than as free nucleic acids. The idea of the approach was to separate mRNPs from cell sap RNases prior to RNA extraction in order to minimize partial enzymatic hydrolysis of mRNAs. The presence of the 5′ terminus (mRNA sense) in cDNAs synthesized was considered as an indication for the suitability of mRNA templates for cDNA synthesis. The cDNAs were synthesized and cloned in lambda gt10 phage. About 300 phages carrying the HN-specific inserts have been identified among 50,000 recombinants, and nine of them were analyzed for the presence of the HN 5′ terminus. It was found that the termini are present in all the clones analyzed. The result is in an agreement with the expectation that removal of cell sap prior to RNA extraction significantly increases the suitability of RNA templates for cDNA synthesis.

Introduction

The integrity of RNA templates is critical for cDNA synthesis. Conventional techniques for preparing viral RNAs are based on the extraction of RNA either from purified viral particles (if virion RNAs are to be utilized as templates) or from

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RNA extraction

Fourteen hours after infection, cells from all the plates were collected, combined, washed with isotonic phosphate buffer saline (PBS), resuspended in 10 mM Tris-HCl, pH 7.6 and 1 mM EDTA (TE), and homogenized in a glass Dounce homogenizer. Nuclei and cell debris were pelleted at a low speed. Cytoplasmic particulate material was separated from the cell sap by centrifugation (SW 41 rotor, 35,000 rpm, 2 hr) of the supernatant from the previous step through 15% sucrose (made in TE). The pellet was resuspended in TE containing 1% SDS, RNAs were released from RNPs by proteinase K digestion (7), and the digest was fractionated overnight (SW 41 rotor, 20,000 rpm, 18°C) through a 15–30% sucrose gradient made in 10 mM Tris-HCl, pH 7.6; 100 mM NaCl: and 1 mM EDTA (TNE) containing 0.5% $NaDodSO_4$. Aliquots of the gradient fractions were analyzed for radioactivity, and 18S peak fractions were combined and precipitated with ethanol. Poly (A)-containing RNAs were separated from the bulk by affinity chromatography (7), and virus-specific poly (A)-contaiing RNA in this final preparation was quantitated by measuring the radioactivity in an aliquot.

cDNA synthesis

cDNA synthesis was performed according to the method of Okayama and Berg (8), as modified by Gubler and Hoffman (9), utilizing an Amersham kit for cDNA synthesis.

Cloning

The cDNA product from the previous step was cloned into the *Eco*RI site of a lambda gt10 phage utilizing the Amersham cDNA cloning kit according to the supplier's protocol. The packaged recombinants were amplified and stored at 4°C as the master library.

NDV hemagglutinin-neuraminidase-specific probe

A NDV-specific fragment (cloned in the *Bam*H1 site of pBR322) that hybridized to the hemagglutinin-neuraminidase (HN) mRNA in a hybridization-selection test was kindly provided by M. Kotler (Hadassah Medical School, Hebrew University, Jerusalem). The fragment was recloned into the *Bam*H1 site of Bluescripts KSP and KSM (Stratagene), and its HN specificity has been confirmed by sequencing both ends (not shown), as described below for cloned cDNA. The fragment was identified by comparing its sequence to that of the reference HN (10)

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cell homogenates or cytoplasmic extracts (if viral mRNAs are to be utilized as templates). The latter approach is usually accompanied by partial degradation of RNAs, probably due to the presence of cell sap RNases in the process of RNA extraction. Obviously, this affects the yields of full-length cDNAs synthesized and usually requires the analysis of large number of clones in order to isolate a desired one. Utilization of RNase inhibitors, though effective during the cDNA synthesis step, is not very helpful during RNA isolation from cytoplasmic extracts or homogenates. Developing an approach that would minimize enzymatic hydrolysis of mRNAs is therefore of obvious interest for those who plan to express or otherwise utilize cloned full-length cDNAs.

It was found in a number of eucaryotic systems that mRNAs are present in cytoplasmic extracts in the form of ribonucleoproteins (mRNPs) rather than free nucleic acids. That is true for mRNAs in animals cells (1–3) and plant cells (4), as well as for virion RNA (5) and virus-specific mRNAs (6). The RNA content in cytoplasmic mRNPs (regardless of their sources) is about 40%, as can be calculated from their buoyant density values (about 1.4 g/ml) in CsCl isopycnic gradients. As far as paramyxovirus-specific mRNAs are concerned, 18S mRNAs are part of the 40S mRNPs (6). Particles of this size can be easily separated from the cell sap by one-step pelleting. The rational of the present research was to attempt to minimize the degradation of Newcastle disease virus (NDV)-specific mRNAs by their extraction from the particulate fraction of cytoplasmic extracts rather than from nonfractionated extracts. It was expected that the 5′ terminus of a model mRNA would be retained in the majority of clones isolated. The results presented show that this was the case.

Materials and Methods

Virus

NDV, strain Australia-Victoria, was propagated in 10-day-old chick embryos and stored as an allantoic virus.

Infection

Thirty chick fibroblast monolayers (in 100-mm Sterilin dishes) were infected with diluted allantoic virus at a multiplicity of infection of 5–10. Four hours after infection, actinimycin D (2 μg/ml) was added to one plate and 30 min later (4.5 hr after infection) ^{3}H-uridine (30 Ci/mmol, 15 μCi/ml) was added to the same plate. The remaining 29 infected monolayers were left nontreated and nonlabeled. The purpose of the labeling was to create an internal marker for 18S virus-specific RNAs.

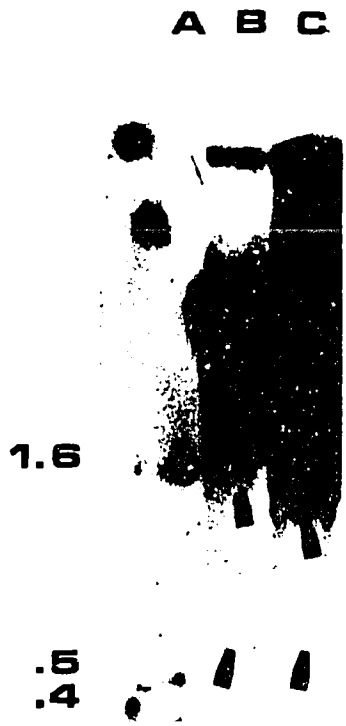

Fig. 1. *Eco*RI restriction of lambda gt10 phage recombinants. Two phages that revealed strong positive hybridization signals with the HN-specific probe were plaque purified and propagated. Extracted DNAs were digested with *Eco*RI, end-labeled with the large fragment of DNA polymerase 1 and ^{32}P dATP, analyzed in a 1% agarose gel, and autoradiographed. *A:* Marker DNAs (*Hin*fI-digested pBR322) of 1631, 515, 396, 344, 298, and 220 bp (sizes of the three largest bands in kbp are shown at the left of the figure. *B:* B52 DNA *C:* B27 DNA. The positions of *Eco*RI fragments are indicated by arrows.

the B52 (after recloning its 1.8 kbp *Eco*RI fragment in a Bluescript) has shown that the suggestion was correct. The remaining six clones contained HN-specific fragments with no internal *Eco*RI sites and probably were generated as the result of insufficient methylation of the cDNA before its linkering and cloning into the phage.

Identification of the end sequences of cloned HN-cDNA

The B27 phage recombinant was partially digested with *Eco*RI with the purpose of isolating molecules that retain internal *Eco*RI recognition sequence(s). Figure 2A

and was found to be the 941 base-long sequence corresponding to the positions 590 through 1530 of the NDV HN gene. The insert was cut off by *Bam*HI, separated from the plasmid by preparative gel electrophoresis, labeled with ^{32}P (either by nick translation or random priming), and utilized as an HN-specific probe for identification of HN cDNA-carrying recombinants in the master library by plaque hybridization.

Sequencing

Selected cDNAs were released from phage recombinants by incomplete *Eco*RI digestion, purified in a gel, and recloned into the *Eco*RI site of the Bluescript KSP plasmid vector. For sequencing of the second strand, the inserts were excised from the Bluescript KSP by double *Bam*HI-*Hin*dIII digestion and recloned into the *Bam*HI-*Hin*dIII-digested Bluescript KSM (see below). Individual DNA strands were rescued from recombinant-carrying cells by R408 helper phage and sequenced by Sequenase (Stratagene) according to the manufacturer's protocol based on the chain termination method (11).

Computer analysis

Computer analysis was done by utilizing the NIH sequencing package (NIH MBUG, ver. 87.1).

Results

Identification of HN-specific clones in the master library

About 50,000 phages of the master library were screened for the presence of HN-specific inserts, and about 300 of them revealed strong positive hybridization signals with the probe. Nine positive clones were propagated, and their DNAs were isolated, digested with *Eco*RI, and analyzed in agarose gels. Inserts with internal *Eco*RI recognition sequences were found in three of them. Figure 1 shows two DNAs (marked B27 and B52), each consisting of two visible fragments: fast (about 0.6 kbp), which are identical for both DNAs and slow (1.3 and 1.8 kbp), which are different for these DNAs. It was concluded that both cDNAs contain at least one *Eco*RI recognition site and are of about 1.9–2 and 2.4–2.5 kbp long, respectively. Since the HN gene is about 2 kbp long and contains internal *Eco*RI recognition sequences (10, 12–15), it seemed likely that B27 contains a full- (or nearly full) length NH cDNA. The insert in the B52 recombinant is even longer, and it was suggested that this is because its 5′ terminus is fused to the 0.5–0.6 kbp 3′-terminal fragment of the upstream F cDNA sequence. Restriction mapping of

Fig. 3. Sequencing the 5′ end of the cloned HN cDNA. The material ("2 kbp" fraction, band 1) from Figure 2 was recloned into the *Eco*RI restriction site of Bluescript KSP and sequenced, as described in Materials and Methods. The plasmid-insert junction is shown.

cDNA strands. The recloned cDNAs have been marked M47 and M52, respectively.

Sequencing the ends of the insert

The cDNAs were sequenced as described in Materials and Methods. Figure 3 shows the insert-plasmid junction corresponding to the 5′ end of the P47 cDNA (mRNA sense). Figure 4 shows the 5′ terminus of this sequence compared to that of the reference NDV HN sequence (10). It is seen in Fig. 4 that the cloned cDNA lacks only seven terminal bases (ACGGGTA) from the very 5′ end of the gene, which suggests that practically full-length cDNA that is equivalent to the gene has been synthesized and cloned. Thus, it seems that all the nine phage recombinants

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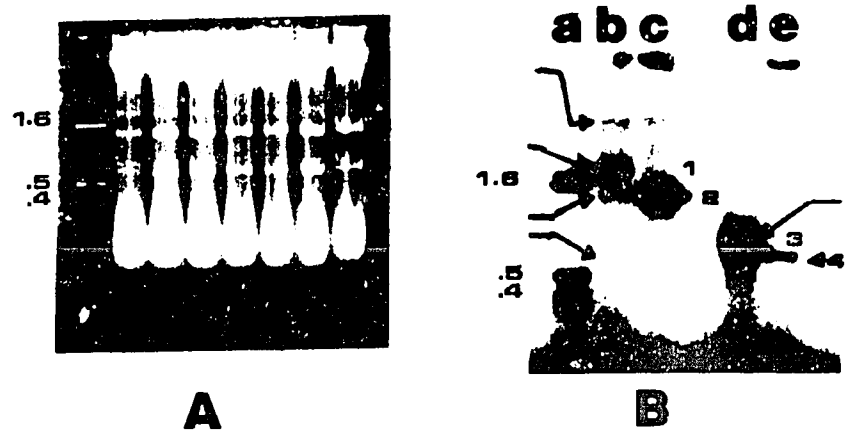

Fig. 2. Partial *Eco*RI digestion of the B27 phage recombinant DNA. *A:* Lambda B27 DNA (3.5 μg per assay) was digested by *Eco*RI for 15, 30, 45, 60, 70 80, and 90 min and analyzed in a 1% agarose gel. Marker DNA, as in Fig. 1, is shown in the left slot. *B:* End-labeling of individual DNAs. Bands 1 (2 kbp), 2 (1.3 kbp), 3 (0.7 kbp), and 4 (0.6 kbp) were cut off separately from the above gel (Figure 2A), eluted, and aliquots of each were individually labeled by the Klenow fragment of DNA polymerase I and ^{32}P dATP. The preparations were electrophoresed in a 1% agarose gel and autoradiographed. a, marker DNA (as in Fig. 1); b, fraction 1; c, fraction 2; d, fraction 3; e, fraction 4.

shows one of the typical kinetic experiments on partial *Eco*RI digestion of lambda B27 DNA. It can be seen that in addition to the 1.3-kbp and 0.6-kbp fragments (bands 2 and 4, respectively) shown in Fig. 1, two more bands are seen in the digest. One of the bands (3) is about 0.7 kbp, demonstrating the presence of an additional *Eco*RI sequence separated from the fragment's end by 100 bp or so. The second band (1) of about 2 kbp probably represents the desired noncut 2-kbp insert (and/or the 1.9- kbp sequence, which lacks the 3′ terminal 100 bases). It was concluded from Fig. 2A that at least two *Eco*RI recognition sites are present in the B27-cloned cDNA. Comparing this to the reference HN cDNA sequence (10) suggests that these sites are located along the isolated fragment (in the 5′ → 3′ direction, mRNA sense) at positions 1299 and 1914, and therefore the cloned cDNA consists of 5′-terminal 1.3-kbp, internal 0.6-kbp, and 3′-terminal 0.1-kbp *Eco*RI fragments. All the bands were eluted and end-labeled separately (with the Klenow fragment of DNA polymerase 1). The result is shown in Fig. 2B. The "2-kbp" preparation, though not homogeneous, was nevertheless utilized for inserting the fragment(s) into the *Eco*RI digested Bluescript KSP. A number of clones carrying a 2-kbp fragment have been isolated. Two of the KSP recombinants (marked P47 and P52) were double-digested by *Bam*HI and *Hin*dIII (these sequences are not present in the cDNA, but flank the *Eco*RI site of the plasmid), and the excised inserts were recloned in the KSM Bluescript (digested with the same restriction enzymes) with the purpose of sequencing the opposite ("minus")

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terminus of the gene. *Eco*RI restriction of cDNA synthesized and cloned in the framework of this study revealed 1.3-, 0.6-, and 0.1-kbp fragments (Fig. 2), thus suggesting the presence of two *Eco*RI recognition sites. Nine clones out of 300 have been studied by restriction analysis, and the large *Eco*RI fragment was found in all of them, suggesting the presence of the 5′ terminus. Moreover, two clones (one of them, B52, is shown in Fig. 1) revealed not only the preserved 5′ HN terminus, but also the presence of 500–600 bases that belong to the upstream F gene (polycistronic mRNAs have been demonstrated in NDV-infected cells—refs. 16 and 17). The presence of HN-F cDNAs is thus an additional indication for the decreased breakdown of the mRNA templates. In some cases, only a large *Eco*RI fragment has been found, which is probably due to nonsufficient methylation of internal *Eco*RI sequences in the cDNA synthesized (this leaves these sequences accessible to *Eco*RI during the *Eco*RI digestion step of cDNA after linkering).

In order to determine the precise sequence of the 5′ terminus in cloned cDNA, one of the clones has been sequenced (Fig. 3) and compared to the reference HN sequence (Fig. 4). It can be seen that all but seven (ACGGGTA) bases, which are a part of the very 5′-terminal sequence and immediately follow the F-NH intergenic dinucleotide (10), are present in the cloned cDNA.

Thus all the nine randomly chosen clones from the cDNA library contain the 5′ terminus of the HN gene. This strongly supports the idea that separation of mRNPs prior to RNA extraction leads to a substantial increase in suitable RNA templates in the final RNA preparation. We cannot exclude, of course, that some cDNAs are also transcribed from partially degraded mRNAs (and thus lack the 5′ mRNA termini), but their number seems to be rather low.

Acknowledgments

Dr. M. Kotler's gift of cloned HN-specific sequence is greatly appreciated. The authors thank the NIH MBUG for the NIH sequencing package which was found to be both reliable and convenient. The research was supported by the Egg and Poultry Board of Israel (grant #847-0157) and AID CDR, USA (grant #5544-G-SS-7006).

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          Reference HN (Beaudette C)        40         50         60
5' ACGGGTAGAACGGTAAGAGAGGCCGCCCCTCAATTGGCAGCCGGGCTTCACAACCTCCGTTCTA
           ::::: :::::::::::::::::::::  ::: ::::::::::::::: ::::::
5' -------gaacgataagagaggccgcccctcaattgcgagctgggcttcacaacctctgttcta
          P47 cDNA            20         30         40         50

     70         80         90         100        110        120
CCGCTTCACCGACAGCAGTCCTCAGTCatgGACCGCGCAGTTAGCCAAGTTGCGTTAGAG--->3'
:::::::::::::::::::::::::::::::::::::::::::::::::::: :::::::
ccgcttcaccgacagcagtcctcagtcATGgaccgcgcagttagccaagttgtgttagag--->3'
  60         70         80   ---  90         100        110
                             MET
```

Fig. 4. Identification of the 5′ end (antigenomic sense) of the HN cloned sequence. The extended sequence from Figure 3 (lower sequence, small letters) was compared to the HN cDNA (upper sequence, capital letters), strain Beaudette C (10), by NUCALN (D.J. Lipman and W.J. Wilbur) of the NIH sequencing package (NIH MBUG, ver. 87.1). The beginning of the single open reading frame (initiation codon ATG) is marked on the sequence.

analyzed retain the 5′ termini of the HN gene, which was directly confirmed by sequencing one of them.

Discussion

The integrity of RNA templates is critical for cDNA synthesis. The amount of full-length cDNA product directly depends on the amount of native RNA extracted from cytoplasmic extracts or cell homogenates. Partial breakdown of RNA probably to a great extent is the result of its enzymatic hydrolysis of cytoplasmic RNases, which retain their activity even in the presence of phenol. The hydrolysis can be somewhat inhibited by adding RNase inhibitors, but usually this is not sufficient to preserve the integrity of extracted RNA. An attempt to separate RNAs from RNases before RNA extraction seems, therefore, to be logical. Since RNAs are present in cytoplasmic extracts of eucaryotes in the form of RNPs, i.e., particles, rather than free nucleic acids (see Introduction), pelleting the particles prior to RNA isolation could result in substantial removal of cell sap RNases. If so, it can be expected that disintegrating RNPs in the absence of the cell sap will decrease the degradation of RNAs, and hence RNAs extracted from the particulate fraction will contain more suitable templates for cDNA synthesis. The presence of mRNAs 5′ termini in the majority of synthesized cDNAs must be the indication for this. The paper describes an attempt to achieve this goal by cloning the NDV HN cDNA as a model.

Though a direct comparison of two cDNAs synthesized on two differently prepared RNA templates has not been made, the efficiency of the method was nevertheless clearly demonstrated. The HN genes of NDV contain one to three *Eco*RI recognition sequences—depending on an NDV strain—among which the 5′ terminus of the gene is the largest (1.3-kbp) *Eco*RI fragment (10,12–15). The presence of such a fragment is therefore the indication for the presence of the 5′

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