Transcriptional Map for Newcastle Disease Virus

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A transcriptional map of Newcastle disease virus was determined by measuring the kinetics of UV inactivation of the transcription of individual genes and of viral infectivity. The inactivation of single genes was monitored by measuring the reduction in the accumulation of viral gene products in vivo and in vitro. In vivo, the accumulation of viral polypeptides in infected cells was measured after reversal of a cycloheximide treatment designed to inhibit secondary transcription. Actinomycin D and a hypertonic medium were used to decrease selectively the synthesis of host cell polypeptides in infected cells. In vitro, mRNA's synthesized by irradiated viruses were analyzed by translation in cell-free systems under conditions in which the amount of each polypeptide synthesized reflected the relative abundance of the corresponding mRNA. UV target sizes were obtained for the genes coding for the HN, F_0, NP, M, L, and P polypeptides; the 47,000-dalton protein was not detected. A comparison of the UV target sizes with the corresponding gene sizes suggested that transcription of these genes initiated at a single promotor and proceeded in the order NP, P, (F_0, M), HN, L. These experiments were performed with Newcastle disease virus strains Australia-Victoria and B1-Hitchner; for both strains, two forms of the P polypeptide which differed in electrophoretic mobility were detected. Proof that the P protein is virus specific was obtained. In addition, infection of chicken embryo cells with avirulent strain B1-Hitchner enhanced the accumulation of at least four polypeptides that appeared to be specified by the host cell rather than by the infecting virus.

Measurement of the sensitivity of a gene to inactivation by UV irradiation can be used to determine the proximity of its promotor site (18). When several genes are transcribed from a single promotor, the order of transcription can also be determined. For several rhabdoviruses and paramyxoviruses, the single-stranded RNA genomes appear to contain a single promotor site (2, 4–7, 15–17, 19), in contrast to the segmented genomes of orthomyxoviruses (1, 39). A complete transcriptional map has been obtained for the rhabdovirus vesicular stomatitis virus (VSV) (2, 4, 6, 7, 14).

The determination of the VSV transcriptional order, in conjunction with physical mapping studies (20), provided considerable insight into the mechanism of transcription by standard and defective particles. The order provided an explanation for the relative amounts of viral gene products which accumulate in vitro and in vivo (45). Measurements of the UV target sizes for transcription of individual genes provided the basis for interpretation of target sizes obtained for viral activities, such as cell killing (33) and inhibition of host cell protein (34) and RNA

syntheses (47). Finally, the VSV transcriptional order was found to correspond to the 3'-to-5' physical order of the viral genes (20).

The UV mapping studies reported in this paper were conducted to investigate the mechanism of transcription, the organization, and the coding capacity of the genome of the avian paramyxovirus Newcastle disease virus (NDV) (9). The genome of this virus is a single negative strand of RNA with a molecular weight estimated by electron microscopy to be 5.1×10^6 to 5.7×10^6 (27). Transcription of the genome in vitro or extraction of RNA from infected cells yields at least six electrophoretically distinct viral mRNA species (5, 12, 13, 26, 42, 44). Translation of the mRNA's in vitro or extraction of polypeptides from infected cells yields polypeptides corresponding to six or seven viral genes (5, 11, 13, 22, 35; G. W. Smith, J. C. Schwalbe, and L. E. Hightower, *in* C. F. Fox, ed., *Biological Assembly*, in press). The following viral polypeptides are known: HN, the hemagglutinin-neuraminidase glycoprotein; F, the fusion glycoprotein; F_0, a glycosylated precursor of F (36, 40); NP, the most abundant protein of the nucleocapsid; P, second in abundance among the core-associated proteins; L, the largest protein of the nucleocapsid; and a 47-kilodalton minor poly-

peptide. The P proteins of NDV have been identified and characterized by G. W. Smith and L. E. Hightower (manuscript in preparation) and by T. G. Morrison (manuscript in preparation). The X polypeptide described by Madansky and Bratt (32) is also a P-related protein (personal communication).

In this work, transcriptional map positions were obtained for the genes coding for proteins NP, P, F_0, M, HN, and L, which together represent most or all of the genome coding capacity. For transcriptional mapping in vivo, a cycloheximide treatment was used to inhibit secondary transcription, which otherwise obscured the polar effects of UV irradiation. In infected cells, viral polypeptides translated from primary transcripts could be detected clearly only by using the avirulent strain B1-Hitchner (*B1*) rather than the virulent strain Australia-Victoria (*AV*). Since strain *B1* has not been well characterized, its polypeptides were identified by comparison with those of the previously studied strain *AV*. In the course of this comparison, it was observed that at least two electrophoretically distinct forms of the NDV P polypeptide accumulated during infections by both strains. Furthermore, infection by the avirulent strain led to an increase in the accumulation of at least four polypeptides that appear to be cell specific, namely, p88, p72, p71, and p23.

(Some of these results were presented at the 79th Annual Meeting of the American Society for Microbiology, 1979, and at the Third Cambridge Conference on Virology, 1977.)

MATERIALS AND METHODS

Virus preparation, assay, and irradiation. NDV strains *AV* and *B1* were grown in embryonated eggs and purified by centrifugation as described previously (13, 22). Some of the virus stocks used in this work were gifts from C. H. Madansky and M. A. Bratt. Plaque assays of virulent (32, 46) strain *AV* were performed on secondary cultures of chicken embryo (CE) cells with an overlay of 0.8% agarose in NCl medium (GIBCO Laboratories) containing 2% calf serum. Avirulent (32, 46) strain *B1* was assayed on monolayers of MDBK cells with an overlay of 0.8% agarose in NCl medium containing 9 µg of trypsin per ml. Preparations of NDV were exposed to UV irradiation as described previously (6). The dose rate was 6.5 ergs/mm² per s.

Cell culture. Secondary cultures of CE cells were prepared as described previously (13; Smith et al., in press). MDBK cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Plaque assays and infections of CE cell cultures were performed at 40°C; MDBK cultures were incubated at 37.5°C.

Conditions for infection, incubation, and labeling of cell cultures. Secondary cultures of CE cells were infected at a multiplicity of 5 PFU/cell

(standard inoculum) or >300 PFU/cell (high-multiplicity inoculum). Beginning with an adsorption period of 45 min, the cultures were incubated at 40°C in NCl medium or Eagle minimal essential medium containing 2% calf serum. In the case of infections with high-multiplicity inocula, virus adsorption and infection before the labeling period were performed in the presence of 50 µg of cycloheximide per ml. For some experiments, cells were also incubated in the presence of 0.5 µg of actinomycin D per ml beginning 4 h before infection and continuing throughout infection before the labeling period.

The labeling period began at 6 h postinfection for cultures infected with standard inocula and at 5 h when high-multiplicity inocula were used. For labeling under standard conditions, cultures were washed well with minimal essential medium containing 10 mM HEPES-hydrochloride (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid hydrochloride) (pH 7.6) and 2.5% (0.38 mg/liter) of the normal methionine content. Cells were then incubated for 30 min at 40°C in the same medium containing [^{35}S]methionine (Amersham Corp.) at a concentration of 30 to 50 µCi/ml. Cells were washed quickly with cold phosphate-buffered saline (pH 7.6) and solubilized in gel sample buffer (28). The extracts were boiled immediately for 2 min. For labeling under hypertonic conditions, cells were washed well with minimal essential medium containing 10 mM HEPES-hydrochloride (pH 7.6), 2.5% of the normal methionine content, and 126 mM additional NaCl. Cultures were incubated in the same medium for 10 min at 40°C. The medium was then replaced by identical medium containing 30 to 50 µCi of [^{35}S]methionine per ml and incubated for 30 min at 40°C. The cells were washed and solubilized as described above.

Preparation of NDV *AV* mRNA's in vitro. Irradiated and unirradiated preparations of NDV were used to synthesize mRNA in vitro in the presence of L-cell cytoplasmic extracts and 0.4% Triton N-101 (13). mRNA was purified by chromatography on columns of oligodeoxythymidylic acid-cellulose as described previously (13). For comparison, mRNA was prepared from reaction mixtures lacking NDV.

Translation of NDV *AV* mRNA's in vitro. Translation of *AV* mRNA's in cell-free systems prepared from mouse L-cells was performed as described previously (13), except that the mRNA concentration was 0.5 µg/ml or less. Reticulocyte lysate reaction mixtures contained 20 mM HEPES-hydrochloride (pH 7.6), 80 mM KCl, 1 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 5.5 mM creatine phosphate, 100 µg of creatine kinase per ml, 50 µM amino acids except methionine, 1 µM methionine (including [^{35}S]methionine at a concentration of 250 µCi/ml), 0.5 mM dithiothreitol, 60 µg of tRNA prepared from mouse L-cells (3) per ml, 25 µM hemin, and 70% (vol/vol) reticulocyte lysate containing 0.05% β-mercaptoethanol. Reticulocytes were obtained from phenylhydrazine-treated rabbits, and lysates were prepared (37). Immediately before the reticulocyte lysates were added to the reaction mixtures, they were adjusted to 1 mM CaCl₂, incubated for 10 min at 20°C with 125 U of *Staphylococcus aureus* nuclease (Boehringer Mannheim) per ml, and then adjusted to 2 mM eth-

ylene glycol-bis(2-aminoethyl ether)-N,N'-tetraacetic acid by using a neutralized stock solution (37). NDV mRNA's made in vitro were added to a final concentration of 0.5 μg/ml or less.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in discontinuous slab gels by the method of Laemmli (28). Electrophoresis was at 20 mA (constant current) for 16 h for 11.5% gels and for 9 h for 7% gels, except as noted below. For autoradiography, gels were acid fixed, dried under a vacuum, and exposed to XR-5 X-ray film. For fluorography, gels were acid fixed, treated for fluorography (30), dried, and exposed to XR-5 X-ray film that had been presensitized to linearize response (31). Quantitation of radioactive bands was by densitometry of the corresponding autoradiogram or fluorogram.

Peptide mapping. Polypeptide bands resolved by SDS-PAGE were excised from the dried gels and analyzed by partial digest peptide mapping according to the procedure of Cleveland et al. (10), using *S. aureus* protease (Boehringer Mannheim). The procedure was as described previously (21, 23), except that the buffers used for treating the gel slices contained 1% (wt/vol) dithiothreitol (29) and SDS-PAGE of the digest fragments was at 20 mA for 8 h.

RESULTS

Comparison of the polypeptides extracted from CE cells infected with strains AV and $B1$. Secondary cultures of CE cells were infected with standard inocula of strains AV and $B1$, incubated for 6 h, exposed to [^{35}S]methionine for 30 min, and solubilized. The radioactive polypeptides were analyzed by SDS-PAGE (Fig. 1). In AV-infected cells (Fig. 1, channel b), the most abundant radioactively labeled species included the following viral polypeptides: L, HN, F_0, NP, M, F_1 (the larger subunit of the F protein [40]), and two electrophoretically distinct forms of an additional viral protein, P, that has not been reported previously (Smith and Hightower, manuscript in preparation; Morrison, manuscript in preparation). The P polypeptides of strain AV were designated according to their apparent molecular weights of 55,000 (P_{55}) and 53,000 (P_{53}), which were obtained by SDS-PAGE in 11.5% gels (Smith and Hightower, manuscript in preparation). The NP, P_{53}, and F_1 species comigrated in the 7% gels shown in Fig. 1. The 47,000-dalton protein (the remaining reported viral protein [11, 22, 42]) was not identified in this work.

The SDS-PAGE pattern of the radioactive polypeptides extracted from $B1$-infected cells (Fig. 1, channel c) resembled the pattern described above for strain AV. However, three differences were noted. First, the NP, P, and M proteins displayed small strain-specific differences in electrophoretic mobility. Second, $B1$-
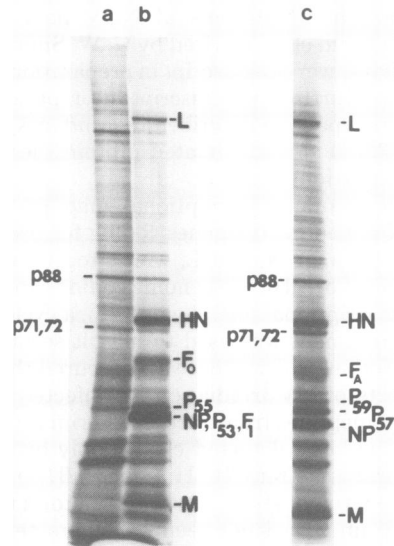

FIG. 1. *SDS-PAGE of polypeptides extracted from secondary cultures of CE cells that were uninfected (channel a) or infected with standard inocula of NDV strains AV (channel b) and $B1$ (channel c). Infected and mock-infected cultures were incubated for 6 h, exposed to [^{35}S]methionine for 30 min, solubilized, and analyzed by electrophoresis in a 7% SDS-polyacrylamide gel. An autoradiogram of the fixed, dried gel is shown. The positions of the major virus-specified and virus-stimulated polypeptides (p88, p72, p71) are marked.*

infected cells accumulated a relatively smaller amount of F_0 and appeared to accumulate no polypeptide corresponding in size in the F_1 glycoprotein of strain AV. However, the SDS-PAGE pattern contained a heterodisperse band of polypeptides (designated F_A) which were intermediate in electrophoretic mobility between the F_0 and F_1 glycoproteins of strain AV. F_A was found to be related to F_0 on the basis of partial digest peptide mapping by the procedure of Cleveland et al. (10) (data not shown). This result confirmed earlier observations of Madansky and Bratt (32; C. H. Madansky, Ph.D. thesis, Harvard University, Cambridge, Mass., 1979). Third, $B1$-infected cells accumulated increased amounts of at least four polypeptides, which were designated p88, p72, p71, and p23 on the basis of their estimated molecular weights. p23 was not retained by the gel represented by the autoradiogram in Fig. 1. p72 and p71 could be separated in 11.5% gels (data not shown). Smaller amounts of polypeptides with the same electrophoretic mobilities were detected in uninfected cells (Fig. 1, channel a) and in AV-infected cells (channel b).
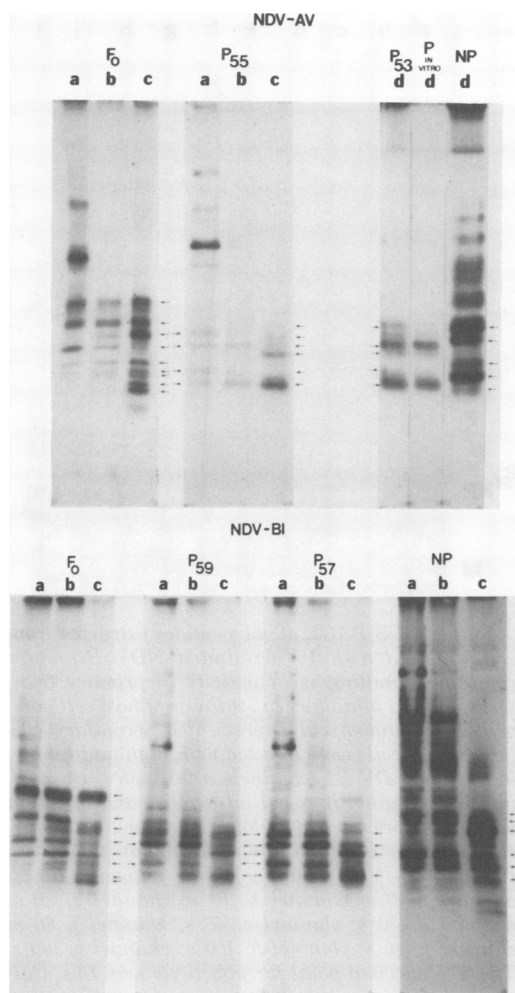
Identification of multiple forms of the P

FIG. 2. *Identification of the P polypeptides of strains AV and B1 by peptide mapping, using the method of Cleveland et al. (10). The following [^{35}S]-methionine-labeled polypeptides were analyzed for strain AV: F_0, P_{53}, and NP from infected cells and P made in vitro, all obtained by SDS-PAGE in 11.5% gels as described in the legends to Fig. 5 and 6; and P_{55} from infected cells, obtained by SDS-PAGE in a 7% gel as described in the legend to Fig. 1. The B1 polypeptides were F_0, P_{59}, P_{57}, and NP, all from infected cells and obtained by SDS-PAGE in a 7% gel as described in the legend to Fig. 1. The viral polypeptides, which were excised from the dried gels, were treated with the following concentrations of S. aureus protease: 0.1 μg/ml (channels labeled a), 10 μg/ml (channels b), 100 μg/ml (channels c), and 25 μg/ml (channels d). The partial digest fragments were resolved by SDS-PAGE in 15% gels. Fluorograms of fixed, treated, dried gels are shown. Some of the characteristic digest fragments are marked with arrows.*

protein. Recently, the NDV P protein has been identified and characterized by analysis of strain AV virions (Smith and Hightower, manuscript in preparation). As Fig. 2 shows, partial digest peptide mapping (10) was used to identify the cellular forms of the P proteins of strains AV and B1 and the AV P protein synthesized in vitro. The P_{55} polypeptide of strain AV and the P_{57} and P_{59} polypeptides of strain B1 were obtained by SDS-PAGE of infected cell extracts by using a 7% gel (Fig. 1). An 11.5% gel was used (see Fig. 5) to obtain the AV P_{53} polypeptide from infected cells and the 53,000-dalton AV P protein that was synthesized in a mouse L-cell cell-free system by using mRNA made in vitro by detergent-activated AV virions. The partial digest mapping patterns obtained for the AV and B1 P polypeptides were very similar (Fig. 2), confirming the identifications. Similar patterns were obtained by partial digest peptide mapping of virion P polypeptides (Smith and Hightower, manuscript in preparation). In contrast, the patterns were different than those obtained for the viral proteins F_0 and NP (Fig. 2), the CE cell polypeptides contained in the same gel region, and the other major viral proteins (data not shown).

Transcriptional mapping of NDV B1 in vivo. Preparations of NDV B1 were exposed to UV irradiation and used to infect secondary cultures of CE cells at high multiplicities of infection; cycloheximide was present to inhibit the replication of undamaged viral genomes. To measure the transcripts synthesized under these conditions, the cycloheximide was removed, the cultures were incubated for 30 min in the presence of [^{35}S]methionine, and the radioactively labeled polypeptides were extracted and analyzed by SDS-PAGE.

Preliminary experiments (data not shown) showed that the accumulation of viral polypeptides under these conditions was severalfold greater in cells infected with the avirulent strain B1 than in cells infected with the virulent strain AV. This was consistent with the observation that avirulent strains synthesize relatively larger amounts of RNA during primary transcription than virulent strains (Madansky, manuscript in preparation). Cycloheximide treatment did not alter the relative abundances or electrophoretic mobilities of most of the viral polypeptides synthesized after removal of the drug. An exception was the F_A polypeptide of the avirulent strain, which could not be detected under these conditions.

Other preliminary experiments (data not shown) were performed to obtain conditions that

facilitated the detection of the viral polypeptides. In cultured cells infected by a variety of viruses, the accumulation of host cell polypeptides can be reduced by increasing the osmolarity of the medium, whereas the accumulation of viral polypeptides is affected to a lesser extent (16, 29). The accumulation of host cell polypeptides was further reduced when actinomycin D was present throughout infection before the addition of hypertonic labeling medium. Thus, for transcriptional mapping, infected cells were treated with actinomycin D, cycloheximide, and hypertonic labeling medium to restrict viral activity to primary transcription and selectively reduce the host cell biosynthetic background (Fig. 3).

The rates of UV inactivation of the individual viral genes were determined by monitoring changes in viral polypeptide accumulation, as measured by densitometry of fluorograms such as those shown in Fig. 3 (Fig. 4). Inactivation of infectivity was measured by plaque assay (Fig. 4). The target for UV inactivation of infectivity was assumed to be the entire viral genome. Based on this assumption, a comparison of the inactivation rate for each gene with the inactivation rate for infectivity provided an estimate of the molecular weight of a UV target for transcription of an individual gene (Table 1; see below). The dashed lines in Fig. 4 show hypothetical inactivation kinetics, which were calculated by assuming that the UV target for each gene was equal to its individual weight. These molecular weights were calculated on the basis of the electrophoretic mobilities of the corresponding protein products (22; unpublished data) and RNA products (12, 26, 42, 49). The measured UV target sizes and estimated gene sizes were compared (Table 1). The UV target size obtained for NP approached the predicted gene size, but for the other genes the measured target sizes were two to five times larger than the sizes predicted and increased in the order NP, P, F_0, M, HN, L. The target size obtained for the L gene was the same as the target size for infectivity.

Transcriptional mapping of NDV AV in vitro. It was possible that the changes in polypeptide accumulation in vivo did not reflect reliably the inactivation of the viral genes. Measurements of the viral mRNA's, the immediate products of transcription, would have afforded a more direct assay. However, the NDV mRNA's have been only partially identified (35, 42), and single-stranded RNAs are difficult to resolve completely. Nevertheless, it is possible to determine relative changes in the accumulation of mRNA's which have been isolated by
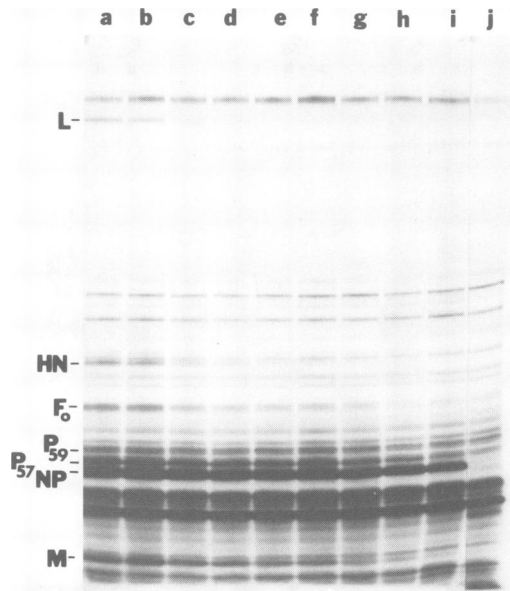

FIG. 3. *SDS-PAGE of polypeptides extracted from cells infected with UV-irradiated NDV B1 under conditions limiting viral activity to primary transcription and inhibitory to synthesis of host cell polypeptides. Actinomycin D-pretreated secondary cultures of CE cells were infected with high-multiplicity inocula of NDV B1 (channels a through i) or mock infected (channel j). Viral adsorption and infection were in the presence of cycloheximide and actinomycin D. Before infection, the virus preparations were exposed to UV irradiation for the following times: channel a, 0 s; channel b, 10 s; channel c, 20 s; channel d, 30 s; channel e, 45 s; channel f, 60 s; channel g, 80 s; channel h, 100 s; channel i, 180 s. The 37% survival point for infectivity was 13 s. Cultures were exposed to [^{35}S]methionine under hypertonic conditions, solubilized, and analyzed by electrophoresis in a 7% SDS–polyacrylamide gel. A fluorogram of a fixed, treated, dried gel is shown. To simplify the figure, some irradiation time points and duplicate points analyzed in the same experiment were omitted. The positions of the major viral polypeptides are marked.*

using oligodeoxythymidylic acid-cellulose and translated in cell-free systems by quantifying the polypeptide products (13).

Preparations of NDV *AV* were irradiated and used to synthesize mRNA in vitro. The transcripts were isolated by oligodeoxythymidylic acid-cellulose column chromatography and assayed for their ability to direct the synthesis of viral polypeptides in cell-free systems prepared from mouse L-cells (Fig. 5) or rabbit reticulocytes (Fig. 6). The concentrations of added mRNA in these experiments were 0.5 µg/ml or less (at least 10-fold lower than the levels that
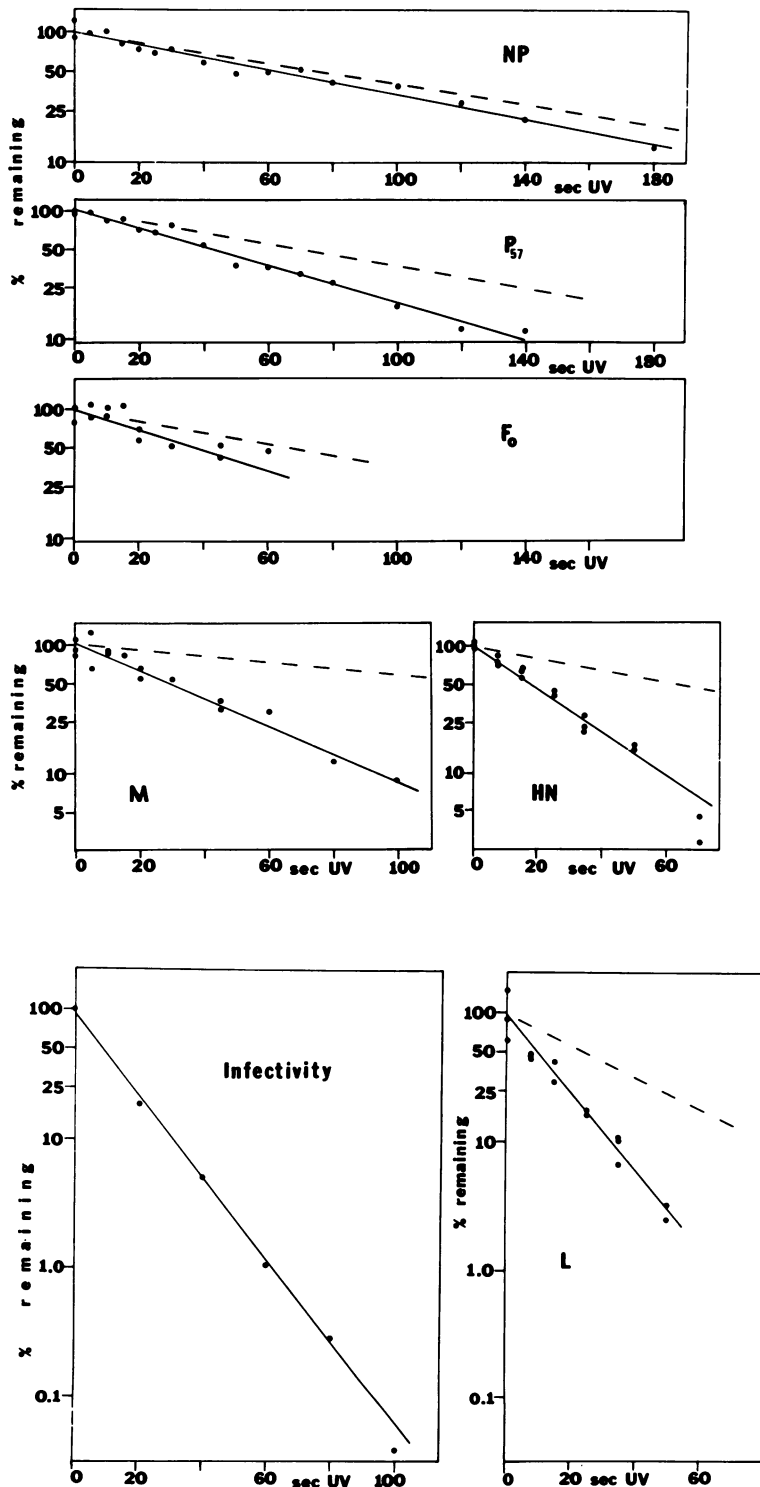
FIG. 4. *Kinetics of reduction of both NDV B1 infectivity and the accumulation of viral polypeptides in vivo as functions of increasing UV irradiation of the virus preparation. Infectivity was determined by the plaque assay. The amount of each polypeptide was determined by densitometry of fluorograms, such as those shown in Fig. 3. For comparison, the dashed lines represent hypothetical inactivation kinetics, based on estimations of gene sizes from the corresponding transcripts or polypeptide sizes (Table 1) and assuming that transcription of each gene initiates at a separate, adjacent promotor.*

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TABLE 1. Comparison of the target sizes for infectivity and individual genes of NDV B1

Viral activity[a]	D^0 (ergs/mm²)[b]	Target size (mol wt, ×10⁻⁶)[c]	Gene size (mol wt) estimated by polypeptide mol wt (×10⁻⁶)[d]	Gene size (mol wt) estimated by transcript mol wt (×10⁻⁶)[e]
Infectivity	91	5.10–5.70		
L protein	91	5.10–5.70	1.98	
HN protein	156	2.97–3.32	0.60	0.83
M protein	267	1.74–1.95	0.36	0.42
F protein	351	1.32–1.48	—[f]	0.77
P protein	429	1.08–1.21	0.51	0.49
NP protein	585	0.79–0.89	0.50	0.70

[a] Inactivation of infectivity and of individual viral genes was monitored as described in the legend to Fig. 4 and in the text.

[b] D^0, Amount of UV irradiation required to reduce activity to 37% survival. The values obtained for four experiments varied over a range of 10 to 15% of the values shown. Similar results were obtained in two experiments with strain AV and one experiment with the avirulent strain N. J. LaSota (data not shown).

[c] The target size for infectivity was assumed to be the molecular weight of the genome, 5.1×10^6 to 5.7×10^6 (27). For individual genes, the target size equalled (D^0 for infectivity/D^0 for individual gene) × genome molecular weight.

[d] Minimum gene sizes based on estimates of the molecular weights of RNAs sufficient to code for the polypeptide gene products, assuming one nucleotide triplet per amino acid. The polypeptide molecular weights were based on the electrophoretic mobilities of the B1 polypeptides. For the HN glycoprotein, the molecular weight of the product made in vitro (unpublished data) was assumed to correspond to the polypeptide portion of the glycoprotein. The following polypeptide molecular weights were used: L, 220,000; HN, 67,000; NP, 55,000; P, 57,000; and M, 40,000.

[e] Gene sizes based on the molecular weights of the corresponding mRNA's were estimated by using the data of Thomas et al. (42) for strain California. A contribution of 130 nucleotide residues was assumed for the polyadenylic acid tail (48) and was subtracted. Direct identifications of the L mRNA (35) and M mRNA (42) have been made. Identifications of the other mRNA's are tentative (6, 42). Band 2 of the polyacrylamide gel pattern of NDV mRNA's was assumed to contain the P protein mRNA. The other tentative assignments are as follows: band 3, NP mRNA; band 4, F mRNA; band 5, HN mRNA.

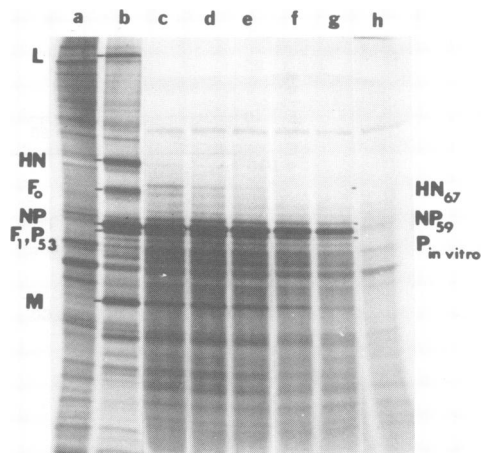[f] A molecular weight estimate for the polypeptide portion of the F glycoprotein was not available.

FIG. 5. SDS-PAGE of polypeptides synthesized in L-cell cell-free systems containing mRNA made in vitro by UV-irradiated NDV AV. Reaction mixtures for transcription included L-cell extracts and no NDV (channel h) or detergent-activated NDV AV that had been exposed to UV irradiation for 0 s (channel c), 25 s (channel d), 50 s (channel e), 100 s (channel f), and 160 s (channel g). The 37% survival point for infectivity was 25 s. The mRNA was purified and added to cell-free systems prepared from mouse L-cells. The [³⁵S]methionine-labeled polypeptides synthesized in vitro were analyzed in an 11.5% SDS-polyacrylamide gel run at 20 mA for 13 h. For comparison, secondary cultures of CE cells were mock-infected (channel a) or infected with a standard inoculum of NDV AV (channel b), incubated, exposed to [³⁵S]methionine for 30 min, solubilized, and run in parallel. An autoradiogram of the fixed dried gel is shown. The positions of the major viral polypeptides synthesized in vitro and in vivo are shown. For AV polypeptides extracted from infected cells (channel b), the P_{53} and F_1 proteins comigrated in a single band under these conditions, with P_{53} in considerably greater abundance.

approach saturation of the translational capacity of the cell-free systems). Therefore, changes in mRNA abundances could be detected reliably by quantitation of the protein products.

The viral polypeptides synthesized by mouse L-cell cell-free systems in response to AV mRNA (Fig. 5) included products corresponding to the following four viral genes: HN, NP, P, and M. The products of the cell-free system have been designated HN_{67}, NP_{59}, NP, P, and M, according to peptide mapping identifications (Fig. 2) (13). For the in vitro products that differed in electrophoretic mobility from the corresponding authentic proteins, subscripts have been added to

indicate their apparent molecular weights (in thousands), based on migration in 11.5% gels (Fig. 6) (13). HN_{67}, previously designated P_{67} (13), appeared to represent an unglycosylated form of the HN protein. NP_{59}, previously designated P_{59} (13), was related to the 56,000-dalton authentic NP. Because NP_{59} was of higher apparent molecular weight, the suggestion was made previously that it might be a precursor of the 56,000-dalton form (13, 23). NP and M made in vitro corresponded to authentic NP and M. The existence of the NDV P protein had not been appreciated previously. The 53,000-dalton AV P protein made in vitro exhibited the same electrophoretic mobility as the F_1 glycoprotein and previously was thought to be an unglycosylated, uncleaved form of the precursor F_0, although this could not be confirmed by our pep-
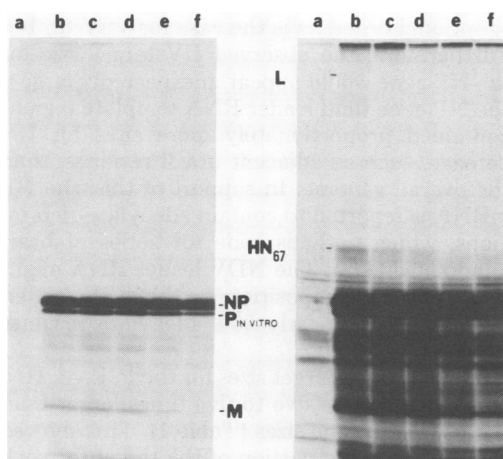
FIG. 6. *SDS-PAGE of [^{35}S]methionine-labeled polypeptides synthesized in a reticulocyte lysate containing mRNA made in vitro by UV-irradiated NDV AV. Two exposures are shown for a single gel that had been acid fixed, treated for fluorography, and dried. Reaction mixtures for transcription included L-cell extracts and no NDV (channel a) or NDV AV that had been exposed to UV irradiation 0 s (channel b), 25 s (channel c), 50 s (channel d), 100 s (channel e), and 160 s (channel f). The same series of transcription reaction mixtures provided the mRNA for the experiments shown here and in Fig. 5. The purified mRNA was analyzed by translation in a reticulocyte lysate, and the protein products were resolved by electrophoresis in an 11.5% SDS-polyacrylamide gel. The positions of viral polypeptides synthesized in vitro are shown.*

tide mapping studies (13). Subsequent mapping studies (Fig. 2) (Smith and Hightower, manuscript in preparation) have established that this 53,000-dalton protein made in vitro is the P protein and is not related to F_0. A cell-free product related to F_0 has yet to be identified. The 53,000-dalton protein electrophoretic species from *A V*-infected cell extracts was previously assumed to contain only F_1, but is now known to be a mixture of P_{53} and F_1, with P_{53} in considerably greater abundance (Smith and Hightower, manuscript in preparation).

The virus-specified polypeptides synthesized by rabbit reticulocyte lysates (Fig. 6) in response to *A V* mRNA were the same as the polypeptides described above except for two differences. First, no distinct NP_{59} band was observed, although in some cases (data not shown) it was possible to detect a heterogeneous series of virus-specified bands resembling NP_{59} in electrophoretic mobility. Second, a polypeptide of relatively low abundance was detected and tentatively designated L on the basis of the following three criteria: comigration with authentic L during SDS-PAGE (data not shown), synthesis in cell-free

systems only in response to NDV mRNA made in vitro (Fig. 6) or extracted from infected cells (data not shown), and a UV sensitivity appropriate for a product of the L gene (Fig. 6). In summary, the polypeptides synthesized in vitro corresponded to five NDV genes, namely, NP, P, M, HN, and L.

Analyses of experiments such as those shown in Fig. 5 and 6 provided rates of inactivation for these five genes and for viral infectivity. Overall, the relative sensitivity to UV inactivation for each NDV gene was the same whether transcription was assayed in vitro or in vivo. The order of increasing sensitivity calculated from experiments performed in vitro was as follows: NP, P, M, HN, L.

DISCUSSION

Recently, the P protein has been shown to be distinct from other viral proteins on the basis of peptide mapping by several methods (Smith and Hightower, manuscript in preparation). Proof that the P protein is virus specific was obtained by synthesis in cell-free systems directed by NDV mRNA made in vitro (Fig. 5 and 6) (13) and by identification of the protein products by peptide mapping (Fig. 2) (Smith and Hightower, manuscript in preparation). Both of the strains analyzed specified the synthesis in vivo of at least two forms of P, but the significance of the multiple forms is not known.

The identification of the F_A polypeptide of the avirulent strain was based on a close similarity to F_0 by partial digest peptide mapping (data not shown) and is in agreement with previous observations (32). F_0 appears to be processed into F_A within 30 min of synthesis (unpublished data; Madansky and Bratt, manuscript in preparation). It is not known whether the shift in electrophoretic mobility reflects a change in carbohydrate or polypeptide content. Interestingly, processing of F_0 into F_A did not occur during a labeling period immediately after cycloheximide treatment (Fig. 3). (Similar experiments [data not shown] without the additional treatments of actinomycin D and hypertonic medium produced the same results.) This might reflect a lack of the processing enzyme(s) under these conditions. Alternatively, since F_A but not F_0 is incorporated into virions (32; Madansky and Bratt, manuscript in preparation) the conversion of F_0 and F_A may be closely coupled to virion morphogenesis, which is blocked by cycloheximide (24).

UV irradiation of NDV preparations resulted in the loss of both infectivity and the expression of individual genes in accordance with single-hit

kinetics. The inactivating hits presumably represent the formation of transcription-terminating uracil dimers. Target size analysis was performed as described previously (5, 6). The rate of inactivation of each gene was determined by measuring the reduction in the accumulation of the corresponding RNA or protein product. This was expressed as a physical target size by comparison with the kinetics of reduction of infectivity, as measured by plaque assay. For animal viruses possessing a small number of genes, it is likely that expression of the entire genome is required to initiate the multiple rounds of infection necessary to form a plaque. Therefore, the rate of loss of infectivity corresponds to a target equivalent to the molecular weight of the genome. A lower rate of inactivation would correspond to a proportionately smaller target. In the case of individual viral genes, the target size is the length of nucleic acid that must be traversed by RNA polymerases in order for expression of a particular viral activity or synthesis of a gene product (18). For comparison, the molecular weights of the NDV genes were estimated according to the molecular weights of the corresponding protein and mRNA gene products (Table 1). The predictions based on transcript size probably provided more accurate estimates, although only the L and M mRNA's have been directly identified (35, 42), and accurate molecular weight determinations for L mRNA were not available. Therefore, gene sizes were also estimated according to the molecular weights of the protein products, assuming one nucleotide triplet per amino acid. These presumably represent minimum estimates, since mRNA's characteristically contain noncoding sequences at both ends.

UV target sizes were obtained for the NP, P, F, M, HN, and L genes. The combined gene molecular weight, calculated by using the transcript sizes estimated by gel electrophoresis when available (Table 1), is about 5.2×10^6. The genome molecular weight is 5.1×10^6 to 5.7×10^6 (27). The target sizes obtained for the NP gene were consistently 10 to 50% larger than the two values for the predicted gene size (Table 1). The physical target for NP gene transcription might be larger than predicted due to contributions from sequences traversed by RNA polymerases but not represented in the NP transcript or polypeptide. For example, transcription might be dependent upon readthrough from a genome region coding for a leader RNA. Banerjee et al. found that NDV transcription yielded a small RNA resembling the VSV leader RNA. The genome region coding for this small NDV RNA presumably precedes the NP gene in the tran-

scriptional order, as is the case for VSV (8, 14). Furthermore, the observed UV target size for the NP gene would appear unexpectedly large if the NP gene (and leader RNA template region) contained proportionately more sites for UV damage, such as adjacent uracil residues, than the overall genome. In support of this, the NP mRNA is reported to contain adenylate-rich regions, which perhaps code for series of basic amino acids (42). The NDV leader RNA might be similar in composition to the VSV leader, which contains nearly 50% adenylate residues (8).

The observed target sizes for the P, F, M, HN, and L genes were two to five times larger than the predicted gene sizes (Table 1). This showed that an extensive portion of the target for each gene consisted of sequences not represented in the transcript. Furthermore, it was evident that the UV targets for the individual genes must overlap extensively, because the combined target size molecular weight was more than twice the genome molecular weight. The simplest model sufficient to explain these results (6, 18) predicts that the NDV genes are contained in a single transcriptional unit equal to the genome molecular weight. The cumulative increases in the UV target sizes suggest that transcription of each gene is dependent upon initiation and readthrough from a single promotor site.

Transcriptional readthrough may explain the existence of several unusual NDV mRNA's made both in vivo and in vitro. Two of the major electrophoretic classes of viral mRNA, designated RNAs 6 and 7, contain both aggregates of smaller 18S mRNA species and large 22S to 24S molecules that resist denaturing conditions (43, 44, 49; G. W. Wertz and P. L. Collins, unpublished data). Other less abundant large nondissociable mRNA's also exist (unpublished data). These large RNAs probably do not represent additional genes, since that would greatly exceed the genome coding capacity. Instead, these large RNAs might be generated by transcriptional readthrough in conjunction with incomplete nucleolytic processing. The same possibility has been suggested by Varich et al. on the basis of hybridization-competition experiments (43, 44).

The target size analysis shown in Fig. 4 and Table 1 indicates that the order of transcription of five of the viral genes is as follows: NP, P, M, HN, L. The relative position of the F gene, although clearly intermediate in the transcriptional order, was not as firmly established. This was because the F-related polypeptide product made in vitro has not been identified and because during SDS-PAGE of infected cell extracts the F_0 glycoprotein comigrated with a CE cell

polypeptide, interfering with measurements of changes in abundance. Our data suggest that the position of the F gene lies between the P and M genes.

The UV target for a gene consists of a single promotor at the 3' target end, any intervening genes, and a coding region at the 5' target end. If the NDV genome consists of contiguous, non-overlapping genes, then each UV target molecular weight should approximate the cumulative molecular weights of the inclusive genes. In this regard there are two apparent discrepancies in the UV target molecular weights shown in Table 1. First, the UV target molecular weights estimated for the P and F genes appear insufficient to contain the expected coding regions. Since the NP, P, and F polypeptides are not related (Fig. 2) (Smith and Hightower, manuscript in preparation), these data raise the possibility that gene overlap occurs in this genome region. Gene overlap appears to occur for fowl plague virus (25). Alternatively, this apparent overlap could reflect inaccuracies in the target size analysis. Second, the difference between the M and HN targets was about 1.30×10^6 daltons, which is considerably larger than the HN gene molecular weight (0.60×10^6 to 0.82×10^6). This might also reflect inaccuracies in the target size analysis. An alternative possibility is that this apparent gap might contain an additional gene, which precedes HN in the transcriptional order. It is possible that the 47-kilodalton NDV structural protein (11, 22, 42) is a product of this genome region. Other paramyxoviruses appear to direct synthesis of a small nonstructural polypeptide (15, 29), which might also correspond to a gene placed between M and HN in the transcriptional order.

The failure to detect the 47-kilodalton protein in this work might be due to substantial changes made in conditions for cell culture and SDS-PAGE since the original report (22). The 47-kilodalton protein is not abundant, and detection might be obscured by the presence of NP fragments and two abundant cellular polypeptides in the corresponding gel region. The identification of viral gene products is complicated by the ability of paramyxoviruses to induce some cellular polypeptides and to incorporate others into virions.

Glazier et al. (17) obtained evidence of a single promotor site for transcription of the Sendai viral genome. However, the gene order which was predicted for Sendai virus (NP, F_0, M, P, HN, L) on the basis of tentative identifications of the mRNA's is different than the order reported here for NDV, primarily in the placement of the P gene, assuming that the NDV and

Sendai P proteins are analogous. The transcriptional orders of NDV (NP, P, F_0, M, HN, L) and VSV (N, NS, M, G, L) are also very similar apart from the F gene, which has no VSV counterpart. Both orders begin with the major nucleocapsid protein NP and end with the largest viral protein (L), which is core-associated for both viruses. The second position in the orders corresponds to another nucleocapsid protein, which may be part of the polymerase complexes. The envelope-associated M proteins and the glycoproteins involved in viral attachment occupy comparable positions intermediate in the transcriptional orders.

Infection of CE cell cultures with avirulent strain *B1* resulted in elevated accumulation of at least four cellular polypeptides, namely p88, p72, p71, and p23. The same species were present in uninfected and *AV*-infected cells, although at lower levels. An analysis of the mRNA's extracted from NDV-infected CE cells indicated that infection resulted in large increases in the amounts of translatable mRNA's for p88, p72, p71, p23, and several other cellular polypeptides (unpublished data). The less dramatic increases in polypeptide amounts shown in Fig. 1 presumably reflect inhibition at the level of synthesis of cellular polypeptides.

The four most abundant NDV-stimulated polypeptides (p88, p72, p71, and p23) are identical to the four most abundant CE cell polypeptides induced by the incorporation of amino acid analogs into proteins (21, 23; unpublished data). Recently, infection of CE cells by the paramyxoviruses Sendai virus and simian virus 5 was found to stimulate the syntheses of p88 and polypeptides corresponding to the two glucose-regulated inducible CE cell genes (38, 41). These observations indicate the existence of a number of inducible cellular genes which code for abundant but unidentified polypeptides that can respond to infection by paramyxoviruses.

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