Replication Cycle of Newcastle Disease Virus in Three Host Cells of Different Permissiveness

By LUCE GRESLAND1, A. NIVELEAU2 AND J. HUPPERT2

1Institut Gustave-Roussy (Groupe de Recherche No 8 du CNRS), 94800 Villejuif and 2Unité de Virologie (U51) Groupe 33, 1 Place du Professeur Joseph Renaut, 69371 Lyon Cedex 2, France

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SUMMARY

Various degrees of permissiveness for NDV have been described in different cell lines. In the present work three systems were investigated: bovine kidney (MDBK) cells, chick embryo (CE) cells and mouse L cells producing 50 to 100 p.f.u., 2 to 10 p.f.u. and less than 0.1 p.f.u./cell, respectively. Analysis of the radioactive virus messenger RNAs (vmRNAs) accumulating in actinomycin D-treated cells throughout the infection cycle revealed that, except for the absence of one 18S vmRNA species in L cells, all vmRNA components were formed in the three cell types, although variations occurred in their total and relative amounts from one cell type to another. Kinetic studies of vmRNA synthesis confirmed the absence of one 18S vmRNA species in L cells and also showed that the labelling rate of this vmRNA component is higher in CE cells. Virus proteins synthesized in the infected cells were labelled with 14C-amino acids and analysed by polyacrylamide gel electrophoresis. All the major NDV polypeptides were formed as expected in both CE and MDBK cells but only traces were detected in L cells. In contrast in a cell-free translation system from wheat germ, all of the NDV major proteins were synthesized using RNA extracted from the three infected cell types. Moreover, the total radioactivity incorporated into NDV proteins was twice as great with CE cell RNA and five times as great with L cell RNA than with equivalent quantities of RNA from MDBK cells.

INTRODUCTION

Newcastle disease virus (NDV) is an avian paramyxovirus capable of infecting cells of other species in vitro. However, according to the origin of the cells, a large variation in virus production may be observed (Wilcox, 1959; Hecht & Summers, 1974; Huppert et al. 1974a). Surprisingly, chicken embryo cells (CEC) in primary or secondary cultures are not very efficient NDV producers, yielding only 2 to 20 p.f.u./cell. Bovine kidney cells (MDBK) are much better producers with yields of 50 to 100 p.f.u./cell. Mouse L-929 cells do not produce more than an average of 0.1 p.f.u./cell and can be considered as practically non-permissive. However, the same virus messenger RNA (vmRNA) species sedimenting at 15S and 18 to 22S are present both in CEC and in L cells. Only the 50S RNA, corresponding to virion RNA present in CEC, could not be detected in L cells (Thacore & Youngner, 1972; Huppert et al. 1974b). We have investigated the mechanism of these variations in permissiveness as a good model for studying the cellular control of virus gene expression.
METHODS

**Virus and cell growth.** Newcastle disease virus (Hertfordshire Strain) was propagated in 11-day-old embryonated eggs of lymphomatosis free chickens (Brown Leghorn – J. Carr’s Strain) bred in our Institute. Purification and titration were as previously described (Huppert et al. 1974a). All cells were grown in minimal essential medium (MEM 0411) obtained from Eurobio-France. Madin Darby bovine kidney cells (MDBK) were grown in MEM supplemented with 5% foetal calf serum and 10% tryptose phosphate broth (TPB). L cells and chicken embryo cells were obtained and grown as previously described (Huppert et al. 1974a). All cell cultures were disrupted with 0.25% trypsin except for MDBK which had to be treated with 0.25% trypsin in 1 mM-EDTA.

**Chemicals.** Actinomycin D was a gift from Merck Sharp and Dohme (Darmstadt, Germany). Acrylamide and N,N’-methylene bisacrylamide from Eastman Kodak Company (New York, U.S.A.) were systematically recrystallized from hot chloroform and acetone solutions respectively. Agarose, electrophoretic grade, was from Bio Rad (Richmond, U.S.A.). Formamide puris. was from Fluka (Buchs, Switzerland) and poly(U)Sepharose from Pharmacia (Uppsala, Sweden).

**Isotopes.** 5'-3H-uridine (sp. act. 20 Ci/mmol) and 14C-amino acids (Chlorella vulgaris protein hydrolysate; sp. act. 45 mCi/Atom of carbon) were obtained from the CEA, (Saclay, France). 2,8-3H-adenosine (50 Ci/mmol) was from NEN (Dreieich, W. Germany). L-35S-methionine (1000 Ci/mmol) was from The Radiochemical Centre (Amersham, Bucks, U.K.).

**Labelling of virus.** Virus was propagated in primary chick embryo cells (CEC) and labelled with 14C-amino acids at a final concentration of 3 μCi/ml for 15 to 16 h. The supernatant was collected and centrifuged on 65% (v/v) sucrose cushions in 0.1 M-NaCl, 0.01 M-tris-HCl, pH 7.5, 0.001 M-Na₂-EDTA (NTE) for 60 min in a Beckman SW25 rotor at 24000 rev/min at 4 °C. Interfaces were collected and diluted with NTE to reduce the sucrose concentration below 20%. Five ml samples were loaded on to a 20 to 65% sucrose NTE gradient and centrifuged for 16 h in a SW25 rotor at 20000 rev/min at 4 °C. Fractions containing the virus were collected and pelleted at 35000 rev/min for 60 min at 4 °C in a SW39 Beckman rotor.

**Labelling and extraction of RNA.** Confluent monolayers were infected with 10 p.f.u./cell. After absorption for 1 h at 4 °C, cells were rinsed and further incubated in MEM containing 4 μg/ml of actinomycin D and 2% calf serum. Incubation was performed at 40 °C in order to shorten the virus growth cycle. In these conditions the cellular metabolism seems not to be modified and the yield of infectious virus remains unchanged when compared to incubation performed at 37 °C (Huppert et al. 1974a). Infected cells were labelled with 3H-uridine (20 μCi/ml) for 1 h at different intervals as indicated in the legends of the figures. Total RNA was extracted according to Clinksales et al. (1977).

**Labelling of intracellular virus protein.** Confluent monolayers were infected as described above and incubated in MEM supplemented with calf serum. Thirty min before labelling the culture medium was removed and a pre-warmed amino acid-free medium without serum was added. At indicated times 14C-amino acids were added at a final concentration of 10 μCi/ml. After 30 min the cells were rinsed with cold 0.1 M-NaCl, scraped off the glass and pelleted by low speed centrifugation. Pellets were treated for polyacrylamide gel electrophoresis as described below.

**Purification of mRNA.** Poly(A)mRNA was purified on a formamide poly(U)-Sepharose column as described by Lindberg & Persson (1972). Eluted poly(A) mRNA was diluted twofold with 0.1 M-NaCl, 0.01 M-tris, pH 7.5 and precipitated with 2.5 vol. of cold ethanol.

**Polyacrylamide gel electrophoresis.** RNA was analysed on 0.5% agarose, 2.5% poly-
acrylamide slab gels according to Peacock & Dingman (1968). Proteins were analysed on 10% polyacrylamide slab gels as described by Laemmli (1970). Samples were treated for 2 min at 100°C with 2% SDS, 5% β-mercaptoethanol and 0.07 M-tris-HCl, pH 7.2. Gels were treated for fluorography as described by Laskey & Mills (1975).

In vitro protein synthesis. A wheat germ extract was prepared according to Marcu & Dudock (1974). Optimum concentrations of Mg²⁺ and K⁺ were 2.5 mM and 100 mM, respectively. The assays (25 μl) contained 5 μg of total cellular RNA, 7.5 μl of wheat germ extract, 20 mM-Hepes, pH 7.5 (adjusted with KOH); 2 mM-DTT, 1 mM-ATP (neutralized with KOH), 20 μM-GTP, 8 mM-creatine PO₄, 40 μg/ml creatine phosphokinase, 2.5 mM-acetate, 100 mM-KCl, 1 μl of L-[³⁵S]-methionine and 25 μM of all 19 unlabelled amino acids (Marcu & Dudock, 1974). Assays were incubated for 90 min at 23°C. Samples were cooled and a sample was counted after TCA precipitation (15 min at 90°C). In vitro synthesis products were analysed by polyacrylamide electrophoresis as described above.

RESULTS

NDV infection of MDBK, CEC and L cells

The time course of virus release and the final yield were determined for each of the three cell types, when infected under similar conditions (see Methods). Cell lysis occurred in MDBK cells between 7 and 9 h p.i. between 9 and 12 h p.i. with CEC and by 20 h p.i. in L cells. The yields were 50 to 100, 2 to 10 and less than 0.1 p.f.u./cell respectively.

RNA labelled with ³H-uridine in the presence of actinomycin D extracted from both MDBK and CEC cells contained three major peaks sedimenting in sucrose gradients at 50S, 35S and 18 to 22S. In L cells the 35S and 18 to 22 S peaks were present but the 50S peak corresponding to the virion RNA was not observed. Chromatography on poly(U)-Sepharose of the labelled RNA extracted from the three types of infected cells indicated that 50 to 60% of the labelled RNA was retained on the column.

Virus messenger RNA (vmRNA) synthesis

Several experiments were performed in order to verify whether the differences in virus yields could be attributed to differences in synthesis or accumulation of vmRNAs. The rate of vmRNA synthesis was estimated in each cell type by plotting as a function of time the specific radioactivity of RNA extracted at intervals from cells infected in the presence of ³H-uridine and actinomycin D. This rate was not significantly distinct in the three cell types studied (Fig. 1.).

RNA from infected cells labelled for 4 h was electrophoresed on agarose–polyacrylamide slab gels. Six components could be distinguished (Fig. 2). We correlate them on the basis of their migration rates relative to the eight components described in the literature (Weiss & Bratt, 1976; Collins et al. 1978; Thomas et al. 1978) as follows, using the letters A to F to avoid confusion: component F would correspond to the 35S component 8; E would represent the 22S components 7 and 6 unresolved; the major component D would be components 4 and 5 migrating together; C would be 3 and components A and B migrating faster than 18S would be equivalent to peaks 2 and 1. All these RNAs contained poly (A).

The most striking difference between labelled RNAs isolated from the three different cell systems was the absence of component A and a relatively high amount of component E in L cells. This may be due to aggregation of A with some larger RNA species. Species A was more abundant in MDBK cells than in CEC. Minor differences appeared also in the relative amounts of components E and C. Furthermore, MDBK and CEC contained some of the 50S virus RNA which was not formed in L cells.

The kinetics of vmRNA synthesis was investigated by pulse labelling the infected cells
Fig. 1. Kinetics of 3H-uridine labelled RNA accumulation in the three types of infected, actinomycin D-treated cells. RNA from: ■—■, CE cells; ○—○, MDBK cells; ●—●, L cells.

Fig. 2. Electrophoresis of vRNA accumulated during 4 h p.i. in the three systems of infected cells. RNA was subjected to a 5 h electrophoresis in 2.5% acrylamide and 0.5% agarose slab gel at 200 V and 4°C. Densitometer tracings were made from fluorographs (see Methods). At the top of the figure, arrows mark the position of the 28S and 18S from ribosomal RNAs. Letters identify the different species of virus RNA as described in the text.

RNA synthesis finished earlier in MDBK cells than in CEC, which is in keeping with the shorter growth cycle. L mRNA synthesis was detected from the first hour p.i. increasing until the end of the experiment (7 h p.i.). In the figures all the previously described RNAs, except the 50S, can be distinguished. Some preparations occasionally contained an additional fast migrating component A' devoid of poly(A) and possibly representing a degradation product. Component A could at no time after infection be detected in RNA from L cells. Component A was relatively more abundant in 60 min pulse labelled CEC than in MDBK cells, whereas the situation was reversed in the 4 h accumulated RNA shown in Fig. 2. This may indicate that RNA A has a shorter half-life in CEC than in MDBK cells. It may also be noted that component E was always more rapidly labelled in L cells than in the other systems.

NDV protein synthesis in infected cells

Infected cells were pulse labelled with 14C-amino acids for 30 min every hour between 2 and 5 h p.i. Solubilized polypeptides were analysed by slab PAGE using polypeptides from labelled purified virions as markers. The bands in the densitometer tracings in Fig. 6 and 7 were in good agreement with the data in the literature (Moore & Burke, 1974; Hightower et al. 1975) if we consider that in our gel system proteins F and NP co-migrate at 56K...
Replication of NDV in different cells

Fig. 3. Electrophoretic analysis of vmRNA synthesized in infected CE cells. Each hour p.i. cells were labelled with $^3$H-uridine for 1 h and the RNA extracted. All RNA samples were subjected to electrophoresis and fluorography as described in the legend to Fig. 2.

Fig. 4. Electrophoretic analysis of vmRNA synthesized in infected MDBK cells. Labelling, electrophoresis and fluorography were as described in the legend to Fig. 3.

Fig. 5. Electrophoretic analysis of vmRNA synthesized in infected L cells. Labelling, electrophoresis and fluorography were as described in the legend to Fig. 3.
components. We regularly observed in the proteins from virions a fast migrating peptide (about 30K) which was absent in infected cells (Inuma & Simpson, 1974). The infected cells contained a 68K polypeptide which probably corresponds to F₀, the uncleared precursor of the virus fusion polypeptides.

No major differences in NDV protein synthesis could be found between MDBK cells except that the synthesis stopped earlier in the former than in the latter (Fig. 6 and 7). In sharp contrast, protein synthesis in L cells, both virus and cellular, was so reduced, that only traces of radioactivity could be detected on several fluorographs (Fig. 8). This indicates a severe inhibition of vmRNA translation in L cells and explains the very small yield of progeny virus observed in those cells.

The low translational activity in infected L cells raised the question of whether the accumulated vmRNA originates exclusively from primary transcription by the polymerase in input virions or whether synthesis of virus coded polymerase escapes inhibition of protein synthesis.

L cells were infected in the presence of ³H-uridine and cycloheximide from either 0 to 2

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**Fig. 6.** Electrophoretic analysis of virus polypeptides synthesized in infected CE cells. Each h p.i. cells were labelled with ³C-amino acids for 30 min. Cells were dissociated with 2% SDS and 5% β-mercaptoethanol for 2 min at 100 °C. Samples were subjected to electrophoresis in a 10% polyacrylamide slab gel for 4 h at 200 V. Densitometer tracings were made from fluorographs (see Methods). The curve at the bottom of the figure represents the separated polypeptides from purified virus. Arrows indicate the position of each virus polypeptide as described in the Results.

**Fig. 7.** Electrophoretic analysis of virus polypeptides synthesized in infected MDBK Cells. Experimental conditions were identical to those described in the legend to Fig. 6.
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Fig. 8. Electrophoretic analysis of virus polypeptides synthesized in infected or non-infected L cells. Experimental conditions were as described in the legends to Fig. 6 and 7.

h p.i. or from 2 to 4 h p.i. in order to inhibit all de novo protein synthesis. When RNA was extracted at 2 h no difference was observed between cycloheximide-treated and untreated cells indicating that synthesis of all virus RNA was due to the virus-associated polymerase. By 4 h virus RNA synthesis was reduced by 80% in cycloheximide-treated compared to untreated cells. Therefore virus induced polymerase was synthesized at 2 h p.i. even in L cells.

NDV protein synthesis in a wheat germ cell-free system

The markedly reduced translation of NDV observed in L cells could be due to a defect in the mRNA. In order to check its functional capacity vmRNA was used to direct protein synthesis in a heterologous cell-free system obtained from wheat germ extracts. Total cellular RNA was extracted from cells 4 h p.i. and the amount of vmRNA present was estimated by calculating the relative radioactivity (Fig. 1). Equal amounts of vmRNA extracted from the various cells were added to the wheat germ incubation mixture. After 90 min at 23 °C the acid insoluble radioactivity was counted and the synthesized products solubilized and electrophoresed on slab gels. In all cases RNA from each of the three cell types directed synthesis of virus polypeptides. Non-glycosylated polypeptides could be recognized by their migration pattern L, HNo*, NP+Fo*, NP1, 47K and M (Fig. 9) according to the nomenclature of Thomas et al. (1978). Furthermore, vmRNA from infected L cells was about five times more efficient in directing translation than those from CEC and MDBK cells (Table 1.)
Fig. 9. Electrophoretic analysis of products of cell-free translation directed by RNA from infected:
(a) L cells; (b) CE cells; (c) MDBK cells; (d) polypeptides from purified virions. Samples were
subjected to electrophoresis and fluorography as described in the legend to Fig. 6.

Table 1. Efficiency in directing translation of vmRNAs from L, CE and MDBK cells

<table>
<thead>
<tr>
<th>Cell system</th>
<th>Radioactivity incorporated in vmRNA (ct/min/μg of total RNA)</th>
<th>Radioactivity incorporated in virus proteins synthesized from 1 μg of total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>7900</td>
<td>33000</td>
</tr>
<tr>
<td>CE</td>
<td>9900</td>
<td>11000</td>
</tr>
<tr>
<td>MDBK</td>
<td>7400</td>
<td>6400</td>
</tr>
</tbody>
</table>

DISCUSSION

Cells from three species had differing degrees of permissiveness for the synthesis of infectious NDV. We attempted to find which step in virus replication is controlled by the cellular regulatory mechanism.

Adsorption and penetration were almost the same in each cell type (our unpublished data), a result supported by the nearly simultaneous appearance of virus mRNA in the infected cells. vmRNA synthesis proceeded at the same rate in all systems. The end of vmRNA synthesis correlates well with the shorter replication cycle of NDV in MDBK cells as compared to CEC. The relative proportion of vmRNAs was not the same in these two cell types, but it is not yet possible to establish a relationship between these variations and the difference in virus production. One particular vmRNA in the 18 to 22S class which migrates on gels faster than the 18S RNA was never detected in L cells. This RNA according to its size could code for the matrix protein M (Collins et al. 1978). However, when we investigated the synthesis of virus proteins, no measurable amount of newly synthesized
proteins, virus or cellular, could be detected in L cells. This finding prompted us to verify whether the vmRNAs produced in large quantities by L cells were functional. Effectively, the addition of the latter RNAs to the cell-free protein synthesizing system directed the synthesis of all non-glycosylated virus polypeptides. Furthermore, the peptide of about 40K corresponding to the M protein was also synthesized in L cells, even though it was supposed to be encoded by the vmRNA which was apparently missing in the cells. This latter finding implies that either the assignment of vmRNAs to polypeptides was not correct or that, in L cells, the information for M protein is included in a larger RNA which is not correctly processed. If so, translation in the wheat germ system could be made possible either by cuts in RNA or by multiple initiation sites on a large RNA molecule. The RNA component E (see Fig. 2 to 5) could be a candidate for such a precursor because it was always found in greater amounts in L cells than in the other cells. Also Thomas et al. (1978) found that RNA components 6 and 7, equivalent to our E component, did not correspond to any specific polypeptide but contained sequences of other vmRNAs. According to the origin of the cells, the vmRNAs have different efficiencies when used to direct protein synthesis in vitro. This may reflect variations in RNA processing (adenylation, methylation etc.) in those cells. The very low level of protein synthesis in L cells is in contrast to the large amount of vmRNA accumulated in those cells. This RNA is synthesized by both primary and secondary transcription, as shown by the cycloheximide experiment. Virus RNA polymerase must therefore be synthesized in L cells even though it could not be detected on gels.

NDV infection interacts with the cellular regulatory mechanisms and provokes an efficient shut-off of protein synthesis. Virus coded proteins are in general resistant to the shut-off except in L cells where no virus protein could be identified. However, in this system some radioactivity was always present in acid-insoluble proteins. The synthesis of virus RNA indicates that at least an active polymerase was synthesized.

Our results show that the properties of the infected cell dictate the amount and proportion of virus components to be synthesized but further studies are needed to elucidate this interaction.

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REFERENCES


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