Virus RNA and Protein Synthesis in Cells Infected with Different Strains of Newcastle Disease Virus

By B. LOMNICZI,* A. MEAGER† AND D. C. BURKE‡

Department of Biochemistry, Marischal College, University of Aberdeen
* Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Budapest, Hungary
† Division of Biological Sciences, University of Warwick, Coventry CV4 7AL, Warwickshire

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SUMMARY

The virus RNAs and proteins synthesized in chick embryo cells infected with different strains of Newcastle disease virus have been characterized by polyacrylamide gel electrophoresis. By comparison of the rates of electrophoretic mobility of virus RNA with those of RNA molecules of known molecular weight, the molecular weights were estimated to be $4.8 \times 10^6$ for the virus particle RNA and $2.5 \times 10^6$, $1.1 \times 10^6$ and $0.4$ to $0.7 \times 10^6$ for the RNA molecules synthesized in infected cells, the latter being heterogeneous. Synthesis of a number of virus particle proteins was readily detected.

INTRODUCTION

Newcastle disease virus is a member of the paramyxovirus group. It contains a molecule of single-stranded RNA with a sedimentation coefficient of 57S (Duesberg & Robinson, 1965) and five or probably six polypeptides, one of which interacts with the RNA to form the virus nucleoprotein, while two others are glycoproteins and are components of the lipoprotein envelope of the virus (Evans & Kingsbury, 1969; Haslem, Cheyne & White, 1969; Caligiri, Klenk & Choppin, 1969; Mountcastle, Compans & Choppin, 1971). Infected cells contain a number of virus RNA molecules (Bratt & Robinson, 1967), including both the virus particle RNA and a series of RNA molecules of lower sedimentation coefficients (18S, 22S and 35S). These have been partially resolved by sucrose gradient centrifugation and shown by hybridization to be complementary to the virus particle RNA (Kingsbury, 1967; Bratt & Robinson, 1967). However, almost nothing is known about the role of these RNA molecules in virus replication, although there is evidence that the 18S RNA is associated with cellular polysomes (Bratt & Robinson, 1967; Blair & Robinson, 1968). In order to establish the role of these RNA molecules more precisely, it was necessary to separate them by a procedure of higher resolving power than sucrose density gradient centrifugation and to establish their molecular weights. The synthesis of the virus proteins was also investigated.

A large number of strains of Newcastle disease virus have been isolated which differ markedly in their virulence for chickens (Waterson, Pennington & Allan, 1967). They can be classed as velogenic (the most virulent), mesogenic (of intermediate virulence) and lentogenic (the least virulent) on the basis of the time taken to cause death in the chick embryo, 1-day-old chicks or adult chickens (Hanson & Brandly, 1955). A number of
properties of the different strains have been examined in a search for a property linked with virulence. These include properties of the virus particle, such as morphology, chemical composition, heat stability, cell-fusing ability, serology and enzymology and also properties of the infected cells, such as speed of virus multiplication (Kohn & Fuchs, 1969; Reeve, Rosenblum & Alexander, 1970; Reeve & Waterson, 1970). None of these characteristics showed any correlation with virulence. However, the capacity to produce plaques and syncytia in chick embryo cells was found to be related to virus virulence (Schloer & Hanson, 1968; Kohn & Fuchs, 1969; Reeve & Alexander, 1970), and a secondary aim of this work was to compare virus RNA and protein synthesis in cells infected with strains of differing virulence in order to obtain more information about the way in which these strains differed. Six strains have been used in this work—two velogenic (Texas and Herts 33), two mesogenic (H and L) and two lentogenic strains (F and LA Sota).

MATERIALS AND METHODS

Materials. Actinomycin D was given by Merck, Sharp and Dohme Ltd. Sodium dodecyl sulphate (specially pure grade) was obtained from British Drug Houses, Poole, Dorset. Ether and phenol were freshly redistilled before use. [5-3H]uridine (30-7 c/m-mole), [3H]DL-valine (250 mc/mmole); [14C]L-valine (260 mc/m-mole) and 32P (orthophosphate in solution containing 1 mg./ml. phosphate buffer pH 7.0) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, and acrylamide, N,N'-methylenebisacrylamide and N,N'N'-tetramethylethylenediamine from Eastman Kodak. Acrylamide and N,N'-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively.

Media and cells. Chick embryo cells were prepared from 9- or 10-day-old embryos as described by Walters, Burke & Skehel (1967).

Virus. The origin, growth and assay of the Newcastle disease virus strains has been previously described (Lomniczi, 1970). Table 1 gives a summary of their biological properties. Growth curves were done in 8×13 cm. flasks containing 25×10⁶ cells. After infection with 15 to 25 p.f.u./cell (for Texas, Herts 33, H and L) or 15 to 25 EID50 (egg infecting dose 50%) for the avirulent strains (F and LA Sota), the cells were washed, incubated at 39°C and small samples removed at intervals for titration. Isotope incorporation experiments were carried out in glass Petri dishes containing 100×10⁶ cells.

Purification of radioactive virus and extraction of RNA. Virus, labelled with 32P, was prepared as described by Barry & Bukrinskaya (1968), except that the density gradient centrifugation was done on a gradient of potassium tartrate (15 to 60%, w/v). When the Texas strain was purified in this way, the product had an infectivity/haemagglutinin ratio one-tenth of that of the crude virus. The RNA was extracted by the method of Bratt and Robinson (1967) and fractionated on 2.2% and 2.5% polyacrylamide gels as described by Peacock & Dingman (1967). Calibration of 2.2% polyacrylamide gels was done using the method of Loening (1967).

Virus protein synthesis in infected cells was measured as described by Joss et al. (1969) Actinomycin (0.5 μg./ml.) was added immediately after infection and the cells pulsed with either [3H]valine (9 μc/culture of infected cells) or [14C]valine (3 μc/culture of uninfected cells) between 4 and 6 hr after infection. The infected and uninfected cells were mixed, nuclei removed by low speed centrifugation, and the proteins in the supernatant solubilized and fractionated by polyacrylamide gel electrophoresis as described by Hay, Skehel & Burke (1968). In some experiments, the isotope was added for 1 hr either 14 or 24 hr after infection. In this case, actinomycin was added 4 hr before addition of isotope.
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Virus RNA synthesis in infected cells was measured as described by Gandhi & Burke (1970). The RNA was precipitated with ethanol at -20°, redissolved and then fractionated on 2·2% or 2·5% polyacrylamide gels as described by Peacock & Dingman (1967).

Preparation of [32P] HeLa cell nucleolar RNA. HeLa cells were grown to confluence (72 hr) in Roux bottles in Eagle’s minimal essential medium containing 7% calf serum and 0·5 mc [32P]/bottle. The cells were removed by trypsinization, pelleted by centrifugation (500 g for 5 min.), and nucleoli were prepared by the method of Penman, Vesco & Penman (1968). Nucleolar RNA was extracted as described by Penman (1966) and Wagner, Katz & Penman (1967).

Table 1. Biological properties of Newcastle disease virus

| Strain     | Mean death time for embryos* (hr) | Cytopathic effect in chick embryo cells† (%) | Plaque size in chick embryo cells‡ (mm.) | ICP§ | IVP|| |
|------------|-----------------------------------|---------------------------------------------|----------------------------------------|------|-----|
| LASOTA     | > 120                             | ≤ 10                                        | —                                      | 0·00 | 0·00|
| F          | > 120                             | ≤ 10                                        | —                                      | 0·00 | 0·00|
| H          | 48                                | ≤ 50                                        | 3 to 4                                 | 0·78 | 0·42|
| L          | 68                                | 100                                         | 1                                      | 0·64 | 0·48|
| TEXAS GB   | 50 to 60                          | 100                                         | 2 to 3                                 | 1·50 | > 1·50|
| HERTS 33   | 50 to 60                          | 100                                         | 2 to 3                                 | 1·50 | > 1·50|

* Using the highest dilutions of virus that killed 100% of the embryos.
† Cytopathic effect was measured after 24 hr at 39°. LA SOTA, F and H caused rounding of cells, L and HERTS 33 rounding of cells and formation of small syncytia, and TEXAS formation of large syncytia.
‡ Measured after 48 hr at 39°.
§ Intracerebral pathogenicity index in 1-day-old chicks (cf. Waterson et al., 1967).
|| Intravenous pathogenicity index in 6-week-old chicks (cf. Waterson, et al., 1967).

RESULTS

Growth curves of Newcastle disease virus strains in chick embryo cells

Measurement of the yield of virus released from infected cells as a function of time showed (Fig. 1) that the two velogenic strains grew most rapidly and to highest yield. The two mesogenic strains yielded less extracellular virus although both produced plaques. The L strain caused an extensive cytopathic effect late in infection, but that due to the H strain never exceeded 50%. The two lentogenic strains (LA SOTA and F) failed to induce plaques or an extensive cytopathic effect, and were therefore titrated in eggs. After a latent period of 4 to 6 hr, titres increased exponentially, reaching maxima 12 to 14 hr after infection. Thus, the kinetics of growth were similar, although the yield of virus released/cell varied between the strains. The relatively poor growth of the H strain and also of strains F and LA SOTA was probably due to autointerference (B. Lomnicz, unpublished results).

Virus RNA synthesis in infected cells

Before investigation of virus RNA synthesis in infected cells by use of polyacrylamide gel electrophoresis, it was necessary to characterize the virus particle RNA. Electrophoresis of the RNA extracted from the TEXAS and F strains gave identical patterns; a single peak being obtained (Fig. 2). Similar results were obtained with the H strain. Electrophoresis in 2·5% polyacrylamide gels failed to demonstrate the low molecular weight material reported by Duesberg & Robinson (1965) which was probably a degradation product.

Infected cells were treated with actinomycin to depress the rate of cellular RNA synthesis
and then pulsed with [3H]uridine before extraction of RNA and fractionation on 2·2% polyacrylamide gels. The results (Fig. 3) showed the presence of multiple species of RNA. These were virus particle RNA (at fraction 7) and RNA species at about fraction 12, fraction 17 and at fraction 25 which probably corresponded to the 35S, 22S and 18S molecules observed by others (Bratt & Robinson, 1967; Blair & Robinson, 1968; Bratt, 1969). Similar results were obtained using 2·5% gels, although in this case virus particle RNA

![Growth curves of Newcastle disease virus strains](image1)

**Fig. 1.** Growth curves of Newcastle disease virus strains. Cells were infected as described in Methods and fluids harvested at the times shown and titrated in chick embryo cells (TEXAS, HERTS 33, H and L) or fertile eggs (F and LA SOTA). TEXAS, • —•••; HERTS 33, ■— ■; H, ○—○; L, ●—●; F, △—△; LA SOTA, ▲—▲.

![Polyacrylamide gel electrophoresis](image2)

**Fig. 2.** Polyacrylamide gel electrophoresis on 2·2% gels of the RNA (○—○) extracted from (a) the TEXAS and (b) the F strain of Newcastle disease virus. Electrophoresis was at 150 V/2 mA tube for 1 hr. The arrows indicate the position of the ribosomal markers (●—● in a).
RNA and proteins in NDV infection

did not enter the gels. All six strains stimulated the synthesis of the same species of RNA, although, as first observed by Bratt (1969), the virulent strains produced somewhat more 18s RNA than the avirulent. Further investigation showed that both the 22s and 18s RNA were heterodisperse (Fig. 4).

Bishop, Claybrook & Spiegelman (1967) have shown that the relative electrophoretic mobility of a series of RNA molecules in polyacrylamide gels was proportional to the log. of the molecular weight. A similar relationship was found (Fig. 4), using the HeLa cell nucleolar RNA (Weinberg & Penman, 1970) and was used to calculate the molecular weights of the virus RNA molecules found in infected cells, which were found to be 0.4
Generally, molecular weights of RNA molecules can be determined from their sedimentation coefficient using the relationship molecular weight = $1550 \times s^2 - 1$ (Spirin, 1963) and the molecular weight values of 18S, 22S and 35S as determined by polyacrylamide gel calibration correspond to those calculated from the Spirin formula. However, the virus particle RNA appears to be an exception, for in this case the widely quoted value of 57S corresponds to a molecular weight of $7.5 \times 10^6$. It is interesting that the molecular weight of 45S RNA from Semliki Forest virus particles ($4.4 \times 10^6$) as calculated from the Spirin formula is approximately the same as that determined by polyacrylamide gel electrophoresis (A. Meager, unpublished observation).

![Image of electrophoretic mobility in 2.2% polyacrylamide gels](image.png)

Fig. 4. The electrophoretic mobility in 2.2% polyacrylamide gels of the HeLa cell $^{32}$P[nucleolar RNA (---), HeLa cell $^{32}$P[ribosomal RNA (---), and $^{3}$H[virus nucleic acid (---) extracted from cells infected with Newcastle disease virus strain TEXAS. The different types of cellular RNA are indicated by the arrows, and the following values were taken for their molecular weights: 45S, 41S and 32S nucleolar RNA, $4.1 \times 10^6$, $3.1 \times 10^6$, and $2.1 \times 10^6$, respectively (Weinberg & Penman, 1970); 28S and 18S ribosomal RNA, $1.65 \times 10^6$ and $0.65 \times 10^6$ (Peterman & Pavlovec, 1966).
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Virus protein synthesis in infected cells

Virus proteins synthesized in infected cells were extracted and fractionated by polyacrylamide gel electrophoresis, using the double labelling procedure of Hay et al. (1968). The results (Fig. 5) are plotted as the $^3$H:$^{14}$C ratios in order to simplify the presentation of the data. The $^3$H and $^{14}$C counts are shown separately for the LA SOTA strain in Fig. 6, and it can be seen that the use of the $^3$H:$^{14}$C ratio removes peaks due to cellular proteins (e.g. at fraction 2 and 25), and also removes any errors due to unequal gel slices (e.g. at fraction 34). The results showed that all six strains synthesized three virus proteins (at fractions 15, 23 and 32) which were almost certainly virus particle proteins (Evans & Kingsbury, 1969; Haslam et al. 1969) and that, in addition, strains L, F and LA SOTA synthesized a fourth virus protein (at fraction 18), which was not detected in the purified virus by Evans & Kingsbury (1969) or Haslem et al. (1969), but may be identical with VP 2 described by Mountcastle et al. (1971). The small peak at fractions 28 to 30 probably

![Fig. 5. Polyacrylamide gel electrophoresis of the proteins extracted from chick embryo cells 6 hr after infection with various strains of Newcastle disease virus.](image-url)
corresponds to the minor virus particle protein found by Mountcastle et al. (1971) in the paramyxoviruses SV 5 and Newcastle disease virus.

Examination of virus protein synthesis at later times in infection showed that cells infected with the LA SOTA strain were still synthesizing virus proteins at 14 hr and 24 hr after infection. A similar result was obtained using the F strain. Virus protein synthesis was still just detectable in cells infected with the H strain 14 hr after infection. Cells infected with more virulent strains did not synthesize any protein, either virus or cellular, at 14 hr after infection, and showed an extensive cytopathic effect. Thus, virus protein synthesis continued longer in cells infected with the avirulent strains.

Fig. 6. Polyacrylamide gel electrophoresis of the proteins extracted from chick embryo strains 6 hr after infection with the LA SOTA strain. The lower panel shows the $^3$H (○—○) and $^{14}$C (●—●) radioactive counts separately.

**DISCUSSION**

Our figure of $4.8 \times 10^6$ for the molecular weight of the virus particle RNA is somewhat lower than previous values, which were generally based on the sedimentation coefficient of the RNA. This varied from 40s for Sendai virus and SV 5 RNA (Barry & Bukrinskaya, 1968; Compans & Choppin, 1968) to 57s for Newcastle disease virus (Duesberg & Robinson, 1965) depending on the conditions used. Using the Spirin formula (1963) these figures correspond to molecular weights of 5.2 and 7.5 \times 10^6. Using an electromicroscopic method, Compans & Choppin (1968) obtained a figure of $6.8 \times 10^6$ for SV 5, while Nakijima & Obara (1967) obtained a figure of $5.8 \times 10^6$ by a chemical method. However, both these latter estimates involved a number of assumptions, certainly more than those involved in the determination of molecular weights from calibrated gels.

The molecular weights of the other virus RNA molecules were $2.5 \times 10^6$, $1.1 \times 10^6$ and
It is interesting that these figures and that for the virus particle RNA, fall in the regular series 0.6:1.2:2.4:4.8. The significance of this is unknown, although it may reflect a subunit structure of the RNA. The greater resolving power of polyacrylamide gel electrophoresis clearly showed that the 18s RNA was heterogeneous. Kingsbury (1970) has pointed that the hybridization data of Bratt & Robinson (1967) demonstrates that either the 18s RNA is heterogeneous with respect to nucleotide sequences, the total population making up sequences complementary to 50% of the genome, or that all the 18s molecules are identical with half the virus genome redundant for their species. The present finding makes the first possibility seem the more likely, and it is interesting that Huang, Baltimore & Stampfer (1970) have found a similar situation in cells infected with vesicular stomatitis virus, a virus that shows a very similar pattern of RNA replication to Newcastle disease virus.

These results give, as yet little clue to the molecular basis of virulence. Virus RNA and protein synthesis were broadly similar although, as reported by Bratt (1969), the relative amount of the different RNA molecules made, varied. It is planned to continue the comparison in a more detailed analysis of virus RNA and protein synthesis.

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REFERENCES


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