The Preparation and Properties of Adenovirus Cores

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SUMMARY

Purified preparations of adenovirus were readily disrupted by heating them for a short time in the presence of sodium deoxycholate. The inner complex of proteins and virus DNA (cores) was partially purified by velocity gradient centrifugation in sucrose or glycerol. Further purification was achieved by equilibrium centrifugation on a preformed gradient of ‘Urografin’. Acrylamide gel electrophoresis showed that the cores contained principally two protein components one of which could be removed at the last stage of purification. The core preparations were infective at an extremely low efficiency, the infectivity being sensitive to DNase, trypsin and to various antivirus sera.

INTRODUCTION

The adenovirus particle contains a number of proteins within the capsid in close association with the virus nucleic acid (Russell, Laver & Sanderson, 1968; Prage, Pettersson & Philipson, 1968; Maizel, White & Scharff, 1968a,b). Various methods have been described for the separation of this nucleoprotein complex or core from the virus capsomeres. These methods have involved the initial disruption of purified virus with acetone (Laver et al. 1968), with urea (Maizel et al. 1968b) or with formamide (Stasny, Neurath & Rubin, 1968). However, in our hands these procedures were not always reproducible with adenovirus type 5. It proved particularly difficult with urea and formamide disruption to stop the reaction before the core proteins were themselves denatured. Our purpose in these studies was to prepare cores which could be examined both biologically and chemically and to this end we searched for a method which was both reproducible and relatively mild. The procedure which we finally adopted involved the disruption of the purified virus by a ‘pulse’ of heat in the presence of sodium deoxycholate and subsequent separation of the released cores on glycerol or sucrose velocity gradients followed by ‘Urografin’ equilibrium gradients. Some of the properties of the cores obtained in this manner are described.

METHODS

Virus. Adenovirus type 5 (Ad 75) was propagated in HeLa or KB cells and purified by extraction of the infected cells with fluorocarbon followed by two or three caesium chloride density gradient centrifugations, as described previously (Russell, Valentine & Pereira, 1967b). The caesium chloride was removed from the purified virus by gel filtration on Sephadex G25.

Radioactive virus. Virus was labelled with $^{32}$Porthophosphate (obtained from the Radio-

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chemical Centre, Amersham, Buckinghamshire) by adding the label to infected cells as described previously (Russell et al. 1968). Čerenkov radiation from aqueous samples (Clausen, 1968) was counted by placing small glass-stoppered vials in empty scintillating vials. The radiation was detected using the tritium settings on the Packard 3375 scintillation spectrometer. Counted in this way, the radioactivity was approximately 30% of that detected by the use of standard scintillation techniques.

**Infectivity titrations.** A line of human epithelial cells (HEK) was used to titrate virus and core preparations. Samples were mixed with HEK cells suspended in Eagle's medium containing tryptophosphate broth and 100 µg./ml. of DEAE-dextran, and shaken at 37°C for 30 min. The cells were then centrifuged (500 g; 10 min.), resuspended in Eagle's medium containing 10% tryptophosphate broth, 1% calf serum and 0.5% carboxymethylcellulose (Russell, 1962) and distributed in Petri dishes. The cells were incubated at 37°C in an atmosphere of 5% CO₂ in air and plaques appeared 8 to 10 days later. In later experiments MgCl₂ was added to the medium to give a final concentration of 20 mM (Williams, 1970). Under these conditions plaques were larger and appeared sooner.

The sensitivity of the core preparations to enzymes was determined by previously incubating the samples with the enzymes in 0.05 M-tris + HCl buffer pH 7.8 at 37°C for 30 min. The effect of various antisera was similarly measured by previous incubation with the samples at 37°C for 30 min.

**Enzymes.** Deoxyribonuclease (DNase crystalline, Sigma Chemical Company) was used at a concentration of 100 µg./ml. in the presence of 25 mM-MgCl₂. Ribonuclease (RNase crystalline, Armour Pharmaceutical Company Ltd) and trypsin (crystalline, BDH Ltd) were used at concentrations of 50 µg./ml.

**Complement-fixation techniques and preparation of antisera** have been described previously (Russell et al. 1967a).

**Density gradients.** Linear sucrose or glycerol gradients were made in 0.005 M-tris + HCl buffer pH 7.8. In most experiments gradients were harvested by puncturing the tubes at the bottom, but in those involving infectivity centrifuge tube contents were harvested from the top. Linear gradients of 'Urografin' (Schering Chemical Company – a 76% aqueous mixture of the sodium and methylglucosamine salts of 3,5-diacetylamino-2,4,6-triiodobenzoic acid) were prepared as 30 to 100% (of the 76% solution) gradients in the dilute tris buffer; samples were placed on top of the 'Urografin' and centrifuged at 90,000 g for 16 hr. A correlation between the density of the 'Urografin' solution and refractive index as measured in the Abbé refractometer was found, namely ρ₂₀ = 3.38In₂₀–3.507.

**Acrylamide gel electrophoresis.** Two systems of gel electrophoresis were used. In the first, electrophoresis was performed in 5% (w/v) acrylamide gels containing 4 M-urea using an acetate + HCl buffer at pH 2.4. Details of this technique were described by Russell et al. (1968). In the second system, samples were dissociated by heating at 100°C for 1 min. in the presence of sodium dodecyl sulphate, mercaptoethanol and urea, and then electrophoresis was carried out in 7.5% (w/v) gels containing sodium dodecyl sulphate and urea. The technique was based on that described by Maizel et al. (1968a) with modifications as described in detail by Skehel & Schild (1971).

**Amino acid analysis.** Samples were hydrolysed in 6N-hydrochloric acid at 105°C for 16 hr and the resultant hydrolysate analysed using standard procedures on the Beckman Amino Acid Analyser.

**Electron microscopy.** The negative staining technique was employed (see Russell et al. 1967b).

**Protein** was determined using the method of Bramhall et al. (1969). Samples precipitated
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Preparation of cores

Russell et al. (1967b) showed that by heating purified virus at 56°C for 1 min. or more, the capsomeres situated at the corners (the pentons) and the surrounding six capsomeres (the peripentonal hexons) could be removed. The resulting complexes of hexons and the virus core readily aggregated, and there was no indication of a clear separation of the virus core from the attached hexons (for explanation of terminology see Ginsberg et al. 1966). However, by heating the purified virus preparations in dilute tris buffer at pH 7.8 in the presence of sodium deoxycholate the cores could be partially separated from the capsids and then further purified by centrifugation. When 32P-virus, after heating in sodium deoxycholate, was placed on a 10 to 40% sucrose gradient and centrifuged at 80,000 g for 1 hr, virus cores as signified by the radioactively labelled virus DNA banded about two-thirds of the way down the gradient (Fig. 1). One-half to two-thirds of the starting radioactivity was recovered in the core fractions. By heat treatment alone, or with sodium deoxycholate alone, all the radioactivity was sedimented. The cores, in contrast to the virus, were extremely sensitive to trypsin, since treatment of the disrupted virus with trypsin (50 μg./ml.) removed all of the radioactivity to the top of the gradient after centrifugation.
A series of experiments determined the optimum conditions for the preparation of cores: no more than 0.75 ml volumes of purified virus suspension in 0.005 M tris-HCl buffer at pH 7.8 containing 0.5% sodium deoxycholate were heated at 56° for 45 sec. in 1 dr. screw-capped vials. The heated vials were immediately cooled in an ice bath. Virus cores have been reproducibly obtained under these conditions on numerous occasions. When opalescent suspensions of virus were used the production of cores was accompanied by a significant clearing of the suspensions, although at very high concentrations of virus a faint blue opalescence still remained. The subsequent enrichment of virus cores was also readily obtained in 10 to 40% glycerol gradients (55,000 g; 1 hr) and the position of the cores in the gradient was detected by determining extinction at 260 and 280 nm. The core fractions still contained a significant amount of hexon antigen (Fig. 2) although most of the antigen, together with the fibre antigen, remained nearer the top of the gradient. The various fractions of the gradient were examined by electron microscopy, and it was apparent that this method of disruption of the virus also yielded the characteristic groups of nine hexons which have been noted previously (Smith, Gehle & Trousdale, 1965; Russell et al. 1967b; Laver et al. 1968). The groups of nine hexons could be seen attached to the core fractions (Fig. 3a), confirming the results of the complement-fixation tests. These groups were more abundant, however, in fractions further up the gradient and at the top fibres and single hexons could also be detected.

Earlier experiments (Russell & Knight, 1967) had suggested that an antigen within the virus could be detected using an antiserum prepared against components formed early in infection (the 'P' antigens). In complement-fixation tests on the various fractions of a core-containing glycerol gradient, using the P antiserum and a hexon antiserum, the P antiserum (which does not react with intact purified virus) reacted to varying degrees with all the fractions, sometimes showing incomplete fixation (Table 1). These findings suggest that the P antigen is indeed exposed during the formation of cores, but that it is also being released from association with the virus DNA, probably changing its conformation in the process.

Further attempts were made to purify the core fractions, and in particular to remove the hexon components. Additional velocity gradient centrifugations in either sucrose or glycerol
Fig. 3. Electron micrographs of negatively stained preparations. (a) Core-containing fraction from a glycerol gradient as in Fig. 2, with high extinction at 260 nm. (b) Core-containing fraction from a ‘Urografin’ gradient.
Table 1. Pattern of complement-fixing antigens after glycerol velocity gradient centrifugation of virus disrupted by heat and sodium deoxycholate

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>$E_{560}$</th>
<th>P antiserum</th>
<th>Hexon antiserum</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/4</td>
<td>1/8</td>
<td>1/16</td>
<td>1/32</td>
</tr>
<tr>
<td>2</td>
<td>0.36</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>0.57</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>2.10</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>2.70</td>
<td>0</td>
<td>±</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.42</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 top</td>
<td>0.64</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

* Antigen dilutions.
The antisera were used at their previously determined optimal dilutions.

4 = 100% fixation; 3 = 75%; 2 = 50%; 1 = 25%.
removed much of the hexon contamination (as judged by complement fixation) but by no means all of it. Equilibrium density gradient centrifugation of the partially purified cores in caesium chloride gave a sharp, flaky band at a buoyant density of 1.41. However, it was obvious that massive clumping and aggregation of the cores had occurred, and no significant purification was obtained. Serological and electron microscopic examination confirmed that the hexons were still present. In an attempt to avoid the problem of hypertonicity associated with the use of caesium chloride gradients, the suitability of gradients formed with ‘Urografin’ was investigated. This material is readily available for urological procedures and has, as a 76% aqueous solution, a density of 1.42 g/cm³. Its main disadvantages are that it is readily precipitated by many organic solvents and solutions of various metallic ions, that it has a very high extinction at 260 and 280 nm., and that it reacts extremely well with the Folin phenol reagent for protein determination (Lowry et al. 1951). When partially purified cores in glycerol (or sucrose) were centrifuged on ‘Urografin’ gradients as described above, a sharp, faintly opalescent band was obtained (at a buoyant density of 1.34), about two-thirds of the way down the gradient. Another sharp upper band (at a buoyant density of 1.24), well separated from the lower band, was noted. Examination of the lower band by electron microscopy showed characteristic clumps of cores (Fig. 3b). Although some aggregation had occurred, this was considerably less than had been noted in the caesium chloride gradients. The upper band contained the groups of nine hexons which had previously been tenaciously attached to the cores.

Since the cores obtained by this ‘Urografin’ procedure contained no hexons detectable by complement fixation, the relevant fractions from the gradients (after u.v. irradiation to inactivate any possible infectivity) were used to prepare anti-core sera in guinea pigs. Unfortunately, only potent anti-hexon sera were obtained; and indeed, detailed examination by electron microscopy indicated that the core preparations were still sparsely contaminated by a few groups of nine hexons. Attempts to prepare anti-core sera by removing these traces of hexon antigen by adsorption with anti-hexon sera have not, so far, been successful.

Some properties of the cores

Polyacrylamide gel electrophoresis of particles of adenovirus type 2 disrupted by sodium dodecyl sulphate has indicated that the virus contains at least nine different polypeptides (Maizel et al. 1968a) and that the nucleoprotein core contains at least three of these polypeptides (Maizel et al. 1968b). It has recently been suggested that the least mobile component in this electrophoresis system is due to aggregated hexon protein and that there are therefore eight significant polypeptides in the virus particle (Horwitz, Scharff & Maizel, 1969; Laver, 1970). Our initial observations used another system of gel electrophoresis involving disruption of the virus in 8 M-urea at pH 2.4 and then electrophoresis in gels containing 4 M-urea at the same pH value (Russell et al. 1968). This method resolved five sharp major bands and a variable number of less mobile components which were evidently aggregates. In our later experiments we have also used disruption by sodium dodecylsulphate and gel electrophoresis procedures, and five major bands of protein were consistently observed. By comparison of the gel patterns obtained with purified hexon, penton, and fibre components we have designated (as in Fig. 4) particular bands in both electrophoresis systems to individual virus components. The principal and least mobile band was due to the major component of the capsid, the hexon; the other components of the capsid, namely fibre and penton base, each contributed one band while there were two bands (as shown below) associated with the core components. These latter two core components were designated core proteins 1 and 2 (Fig. 4). Other minor bands, which were not reproducibly detected, were disregarded;
they could perhaps be degradation products of the other components (Pereira & Skehel, 1971).

Using both these systems of electrophoresis, analysis of the cores produced in glycerol gradients (in agreement with the serological and electron microscopical observations) indicated that a significant amount of hexon polypeptide was still present together with the core proteins 1 and 2 (Fig. 4). By further purification in 'Urografin' gradients, the cores, rather surprisingly, showed only the presence of core protein 2. In some preparations a trace of hexon could sometimes be detected on the gels, but never any indication of core protein 1.

Fig. 4. Stained acrylamide gels after electrophoresis of purified virus and core preparations in urea at pH 2.4 and in sodium dodecyl sulphate at pH 7.2.
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This result indicated that one of the core proteins was more intimately bound to the virus DNA than the other. Molecular weight determinations of the various polypeptides were made using sodium dodecyl sulphate acrylamide gel electrophoresis with suitable protein standards (Shapiro, Vinuelo & Maizel, 1967) and in general the results agreed with those already published by Maizel et al. (1968b). In particular, we obtained values of 46,000 and 22,000 respectively for the mol. wts of core proteins 1 and 2. In this connexion it was of interest to ascertain how much protein was left in association with the virus DNA, particularly in the cores prepared after centrifugation in ‘Urografin’. Three different experiments indicated a ratio of protein to DNA of 1·1 ± 0·2 and since the virus DNA has been shown to be present in the virus particle as a single molecule of mol. wt 23 × 10^6 (Green et al. 1967) this result implied that of the order of 1000 polypeptides of core protein 2 were associated with each virus DNA molecule.

Table 2. Amino acid analyses of adenovirus type 5 and of core preparations

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Virus</th>
<th>Cores from glycerol gradients</th>
<th>Core protein-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>3·6</td>
<td>3·7</td>
<td>3·5</td>
</tr>
<tr>
<td>Histidine</td>
<td>1·6</td>
<td>1·9</td>
<td>2·2</td>
</tr>
<tr>
<td>Arginine</td>
<td>7·7</td>
<td>11·6</td>
<td>22·2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>12·0</td>
<td>10·6</td>
<td>6·1</td>
</tr>
<tr>
<td>Threonine</td>
<td>6·7</td>
<td>7·4</td>
<td>6·2</td>
</tr>
<tr>
<td>Serine</td>
<td>6·8</td>
<td>6·2</td>
<td>3·2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9·9</td>
<td>7·1</td>
<td>3·4</td>
</tr>
<tr>
<td>Proline</td>
<td>5·7</td>
<td>5·8</td>
<td>5·9</td>
</tr>
<tr>
<td>Glycine</td>
<td>8·6</td>
<td>8·1</td>
<td>11·2</td>
</tr>
<tr>
<td>Alanine</td>
<td>10·1</td>
<td>12·3</td>
<td>18·7</td>
</tr>
<tr>
<td>(\frac{1}{2}) Cystine</td>
<td>0·7</td>
<td>0·4</td>
<td>Tr</td>
</tr>
<tr>
<td>Valine</td>
<td>4·6</td>
<td>6·5</td>
<td>9·5</td>
</tr>
<tr>
<td>Methionine</td>
<td>1·4</td>
<td>1·9</td>
<td>1·3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4·3</td>
<td>3·3</td>
<td>2·3</td>
</tr>
<tr>
<td>Leucine</td>
<td>8·7</td>
<td>6·8</td>
<td>2·6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3·5</td>
<td>3·6</td>
<td>1·9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4·0</td>
<td>3·0</td>
<td>0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Acidic/basic</td>
<td>1·70</td>
<td>1·03</td>
<td>0·34</td>
</tr>
</tbody>
</table>

ND = not determined; Tr = Trace.

The results are expressed as moles/100 moles of amino acids recovered (ammonia not being included in the total). No corrections have been made for possible loss of amino acids during acid hydrolysis.

Amino acid analyses were made on purified virus, on cores obtained after glycerol gradient centrifugation and on the cores obtained after ‘Urografin’ gradient centrifugation. The core-2 protein, in contrast to that of the virus and of the partially purified cores, was extremely basic (Table 2).

Several infectivity assays were performed using cores prepared by several cycles of velocity gradient centrifugation. The plaque technique in the presence of DEAE-dextran very little infectivity was observed in all these preparations. The efficiency of this infectivity was of the order of 10^{-8} of that of the intact virus. It could be distinguished from that of the virus because it required the presence of DEAE-dextran, it was significantly sensitive to deoxyribonuclease, to even low concentrations of trypsin, and to the P antiserum, all of which have no effect on normal virus infectivity. It was also notable that the infectivity was partially susceptible to treatment with antisera to type 5 hexon but not to antisera to type 2 hexon. The dose response of the assay showed that the infectivity was not governed
by a single-hit process, suggesting that multiplicity reactivation and aggregation were of significance in the process.

In one experiment the loss of infectivity during the purification procedures was analysed. Thus, a purified virus suspension containing a total of $3 \times 10^{10}$ p.f.u., after disruption by heat and sodium deoxycholate and subsequent enrichment of cores on a glycerol gradient, yielded only $3 \times 10^4$ p.f.u., and after further purification on a ‘Urografin’ gradient only 200 p.f.u. could be recovered.

**DISCUSSION**

The internal proteins of the adenovirus account for at least 20% of the virus protein (Laver et al. 1968). Are they merely structural components, allowing the DNA to be folded up before being enclosed in a protective capsid, or do they play some more essential role in the process of infection? To answer these questions, a reproducible method of preparing the cores is clearly necessary, and the methods described in our experiments offer one approach to this problem. Previous studies have shown that these components possess many of the properties of the histones and protamines: they are acid-soluble, apparently rich in arginine, possess an N-terminal alanine and are closely associated with the virus nucleic acid (Russell et al. 1968). The present investigation confirms the arginine-rich nature of the core components and also, possibly as a result of this fact, emphasizes the difficulties involved in the manipulation of this nucleoprotein. The isolated cores often aggregated in the absence of sodium deoxycholate or when the ionic strength of the solutions was increased, and they were prone to stick readily to glass surfaces and to dialysis tubing. Furthermore, both the analysis of the distribution of antigens on velocity gradients after disruption by heat and sodium deoxycholate and the acrylamide gel electrophoresis experiments suggested that the adenovirus core probably does not exist as a stable entity – a conclusion also obtained from studies of infected cells (Mäntyjärvi & Russell, 1969).

In the light of these findings, it is important to reconsider what is meant by the term ‘adenovirus core’. Originally, the term was applied to the complex of virus DNA and proteins other than those found in the capsid (e.g. as in Laver et al. 1968). If this definition is accepted, the core consists of virus DNA + core proteins 1 and 2 (neglecting possible minor components). Our experiments have shown, however, that core protein-1 is readily removed from this complex leaving the virus DNA in close association with core protein-2. This nucleoprotein has been reproducibly obtained and appears to be relatively stable; we suggest that in future this complex of virus DNA and core protein 2 should be referred to as the *inner nucleoprotein* of the adenovirus.

The amino acid analysis of core protein 2 shows a very unusual distribution of amino acids, arginine (22%) alanine (19%) and glycine (11%) contributing more than half of the total amino acids. The apparent absence of phenylalanine is noteworthy. This result agrees extremely well with an analysis recently published by Laver (1970) who eluted the corresponding polypeptide (band 8) from an acrylamide gel after electrophoresis of the purified type 2 adenovirus. This distribution of amino acids shows some similarities to those of the protamines, and in particular to some of the mammalian sperm proteins, and is significantly different from those of the histones. On the other hand, the molecular weight of core protein 2 (as determined by the sodium dodecyl sulphate acrylamide gel method) is considerably greater than those of the protamines and similar to that of the arginine-rich F 3 histone (cf. Hnilica, 1967). These findings thus indicate that this basic protein cannot be readily assigned to either of these two major classes of basic proteins. The two more minor bands which are consistently observed (but at variable relative concentrations to the other components) in
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acrylamide gels electrophoresed in urea at low pH values when analysing purified virus or cores and which have a similar electrophoretic mobility to standard protamines (Russell et al. 1968) are not seen when the sodium dodecyl sulphate acrylamide gel technique is used (cf. Laver, 1970).

We were disappointed not to be able to prepare antibody to the core proteins. Our failure probably reflects the extremely poor antigenicity of these core proteins and the correspondingly potent antigenicity of the hexon antigen which was always found in close association with the cores.

The infectivity studies showed that the cores are infective but at an extremely low efficiency and only when adsorbed to the cells in the presence of DEAE-dextran. The tendency of the core components to aggregate readily was also demonstrated in the infectivity assays, and this made quantitative comparisons between different experiments rather difficult. However, it is clear that the cores were sensitive to trypsin as well as to deoxyribonuclease, suggesting an essential role for the protein components of the cores in the infective process. It was also significant that the virus nucleoprotein preparations were even less infective. In support of this finding we have been unable to demonstrate any infectivity associated with the isolated virus DNA (unpublished observations). This is in contrast to the observations of Burnett & Harrington (1968) who found that the DNA extracted from simian adenovirus SA7 was infective, although they did not test its trypsin sensitivity. It is not known what part the small amount of contaminating hexon plays in the infective process, and it may be significant that the infectivity was also partially sensitive to the homologous anti-hexon serum.

The addition of DEAE-dextran to the DNAs of smaller viruses has been an effective means of introducing them into the cell and in most cases allows a relatively efficient infectious process to occur (cf. Pagano, 1970). Our results, on the other hand, tend to suggest that the adenovirus DNA is not infective per se and further that the virus proteins are not merely a means of wrapping up the nucleic acid. Possibly these proteins fulfil some other critical functions which allow the genetic message to be transcribed in an efficient fashion.

Infectivity with similar characteristics has been described by Dubes, Roegner & Cronn (1968). By heating adenovirus at 70° these authors could demonstrate residual infectivity which depended on the presence of DEAE-dextran and was sensitive to both deoxyribonuclease and trypsin.

Further work is now being done on the biological and possible enzymological properties of the core and inner nucleoprotein in an effort to determine their function in virus infection.

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REFERENCES


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