The Polypeptides of Adenovirus Type 12

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SUMMARY

Six polypeptides could be detected in purified preparations of adenovirus type 12 by polyacrylamide gel electrophoresis. One of these polypeptides (the Y polypeptide) could not be seen in type 5 virus preparations and appeared to be associated with the core components rather than with the capsid components. Incomplete particles of type 12 virus, while lacking the core polypeptides contained three other polypeptides. The synthesis of the structural polypeptides could be detected in infected cells by labelling with $^{35}$S-methionine; in addition, there were at least eight other polypeptides which appeared to be specific for the infected cell (ICSP's). One of these, ICSP-3, of mol. wt. 55,000, was synthesized early in infection even in the presence of an inhibitor of DNA synthesis and was thus presumed to be related to the adenovirus type 12 tumour (T) antigen. Phosphorylation of polypeptides was noted in the infected cells and in the purified virus both fibre and core-1 polypeptides appeared to be phosphorylated.

INTRODUCTION

Polypeptide synthesis in virus-infected cells can be effectively monitored by pulse labelling cells with $^{35}$S-methionine followed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and autoradiography (Russell & Skehel, 1972; Skehel, 1972). Using this method a line of human cells infected with adenovirus type 5 has been shown to synthesize five structural polypeptides and at least five other polypeptides which have been designated infected cell specific polypeptides (ICSP's) 1 to 5.

Adenovirus type 12, in contrast to type 5, can readily induce tumours in baby hamsters and many experiments to compare the properties of these two types have been made in an effort to pinpoint the factor(s) responsible for the oncogenic properties of the type 12 virus (Huebner, 1967; Schlesinger, 1969).

In attempts to obtain further information on this problem a comparative study of the polypeptides synthesized in cells infected by these two types of adenovirus was undertaken.

METHODS

Virus and cells. Adenovirus type 5 (Ad 75) was propagated in either KB or HeLa cells as described previously (Russell et al. 1967). Type 12 virus (Huie) was grown in secondary monolayer cultures of human embryo kidney cells in Eagle's medium as described before (Russell & Bomford, 1970).

Virus unlabelled and labelled with $^{35}$S-methionine and $^{32}$P-orthophosphate was
purified by procedures previously described (Russell & Skehel, 1972; Russell et al. 1972). Cells infected with type 12 virus in contrast to those with type 5, produced significant amounts of top component which was seen as a discrete light scattering band immediately above the opalescent virus band in the velocity caesium chloride gradient of the initial purification schedule. Further purification of the top component was obtained by density gradient centrifuging in caesium chloride followed by velocity gradient centrifuging through a 10 to 40 % glycerol gradient (60 min, 70000 g) onto a pad containing caesium chloride of density 1.45 g/ml in 40 % glycerol. Density gradients were determined by measuring the refractive indices of individual drops at various points in the gradient, using an Abbe refractometer.

Infection of cells and labelling with isotopes. Secondary cultures of human embryo kidney cells in test tube cultures were infected with type 5 or type 12 adenovirus at an added multiplicity of about 10 p.f.u./cell. Replicate cultures containing approximately 5 × 10^5 cells were then labelled for 1 h periods with either high specific activity [35S]-methionine or [32P]-orthophosphate (both obtained from the Radiochemical Centre, Amersham, Bucks) and the cells then harvested and processed as described previously (Russell & Skehel, 1972; Russell et al. 1972).

Polyacrylamide gel electrophoresis and autoradiography. Techniques were as previously documented (Russell, McIntosh & Skehel, 1971; Russell & Skehel, 1972), using 7.5 and 10 % polyacrylamide gels. Purified [35S]-labelled type 5 virus was used as reference in most experiments.

Densitometer tracings on the autoradiograms were made using a Joyce Loebl integrating microdensitometer.

Electron microscopy was carried out using negative staining with sodium silicotungstate (Nermut, 1972).

RESULTS

Polypeptide analysis of purified virus

Preparations of type 5 and type 12 virus labelled with [35S]-methionine were purified and then analysed by SDS polyacrylamide gel electrophoresis. Fig. 1 A shows the autoradiograms after electrophoresis and it can be seen that the type 12 virus preparations contained polypeptides of similar mobilities to the previously identified hexon, fibre and core polypeptides of type 5 virus (Russell et al. 1971). However, a polypeptide of type 12 virus cannot be seen in the same position as the penton base polypeptide of type 5 virus but another less intense band can be noted at a higher mobility, slightly slower than the fibre polypeptide. There is, moreover, another polypeptide (designated the Y polypeptide) in type 12 virus between the core-1 and core-2 polypeptides which is not in evidence in type 5 [35S]-labelled virus (or in unlabelled type 5 virus electropherograms stained with Coomassie blue (Russell, 1971; Russell et al. 1971). In attempts to ascertain if the extra polypeptide could be ascribed to any of the known components of the virus, preparations of purified unlabelled type 12 virus were dialysed against distilled water at 4 °C overnight and then given a low speed centrifuging (30 min, 300 g). This procedure has been successful in releasing pentons from purified preparations of types 2 and 5 adenovirus (Laver, Wrigley & Pereira, 1969). Examination by electron microscopy of the supernatant fluid after dialysis and centrifuging revealed the presence of mainly isolated fibres and pentons and possibly some hexons (Fig. 2). The polypeptide pattern derived from this preparation after electrophoresis and staining with Coomassie blue is shown in Fig. 1 B along with that derived from the purified virus (Fig. 1 B). This pattern is consistent with the presence of fibre and hexon polypeptides and
Polypeptides of adenovirus type 12

Fig. 1. (A) Autoradiograms of [35S]-methionine labelled purified type 12 (left) and type 5 (right) viruses, after SDS polyacrylamide gel electrophoresis. Assignment of the various structural polypeptides of type 5 virus are shown on the right and the Y polypeptide of type 12 virus on the left. (B) Electropherograms of purified type 12 virus (left) and of the supernatant fluid after dialysis of the purified virus and subsequent sedimentation (right) (Coomassie staining).

the minor band again noted at mobilities slightly slower than the fibre polypeptide is presumably derived from the type 12 penton base (this band is only just discernable in the electropherogram of the purified virus but is not clearly reproduced in the print in Fig. 1 B). The Y polypeptide band can be seen in the stained gels of the purified virus although it appears to be less intense than in the autoradiograms; it also stains with a more reddish tinge than the other bands. These results suggest that the Y polypeptide is not associated with either the penton base or fibre components of the virus and it is presumed to be associated either with hexons or the core components.

Polypeptide analysis of ‘top’ component

Cells infected with type 12 virus, in contrast to type 5 virus, produce significant quantities of virus particles which are deficient in nucleic acid and hence have a lower density in
equilibrium gradients. Preparations of the 'top' component and virus particles were obtained from \(^{35}S\)-infected cell extracts and separated and purified by velocity and equilibrium density gradient sedimentation in caesium chloride. Fig. 3 shows the distribution of radioactive label in an equilibrium caesium chloride gradient and illustrates the clear separation of the two species, the 'top' component, banding at a density of 1.32 in contrast to the complete particles which have a bouyant density of 1.35. On examination by electron microscopy, typical incomplete particles which allowed penetration of the negative stain, were seen in the 'top' component fractions (see insets to Fig. 3). These preparations were analysed by SDS polyacrylamide gel electrophoresis followed by autoradiography, and densitometer tracings from the resulting autoradiograms are shown in Fig. 4. In both preparations comparable amounts of hexon and fibre polypeptides can be clearly seen; the presumed penton base polypeptide can be discerned in the complete particles but is not obvious in the incomplete particles. It is also evident that both core polypeptides are absent in the incomplete particles. Polypeptide Y can be seen in the incomplete particles but the stoichiometry relative to both hexon and fibre polypeptides is very much reduced thus suggesting that polypeptide Y is associated with the core rather than with the capsid components. In addition, three other polypeptides labelled \(\alpha\), \(\beta\) and \(\gamma\) can be discerned in the incomplete particles. Further purification of both the incomplete and complete particles by glycerol velocity sedimentation on to a cushion of caesium chloride, did not change the polypeptide patterns of the particles. This finding suggests that polypeptides \(\alpha\), \(\beta\) and \(\gamma\) are not merely nonspecific cellular contaminants, since these centrifuging conditions would only sediment virus particles on to the caesium chloride cushion, groups of hexons and smaller particles being retained towards the top of the gradient (Russell et al. 1971). The three polypeptides \(\alpha\), \(\beta\) and \(\gamma\) were consistently detected in a number of different preparations although their relative stoichiometry and that of the Y polypeptide varied. Similar results
Polypeptides of adenovirus type 12

The synthesis of polypeptides in virus infected cells

The synthesis of polypeptides in cells infected with adenovirus type 12 was examined by pulse-labelling human embryo kidney cells with $[^{35}S]$-methionine at various times after infection and then comparing the polypeptide patterns produced after SDS polyacrylamide...
gel electrophoresis and autoradiography with those obtained from parallel cultures infected with adenovirus type 5. Fig. 5A shows that by 30 h after infection with type 12 virus, polypeptides corresponding in mobility to the structural polypeptides of the complete particles can be observed but there are at least eight other polypeptides which appear in the infected cell and cannot be recognized in the uninfected cell (see below and Fig. 6). These polypeptides can thus be termed infected cell specific polypeptides (ICSP's). Only five of these polypeptides were noted in type 5 infection (Russell & Skehel, 1972) and the additional polypeptides seen in type 12 infection have mobilities between the fibre and core-2 polypeptides. A distinctive feature of the type 12 infected cells, however, is a heavily labelled band (designated 1-3) which can be observed in the autoradiograms early in infection at a mobility slightly greater than the fibre polypeptide. (The fibre polypeptide can be just discerned in the 30 h sample). Better resolution of the larger polypeptides was obtained by analysis in 7.5% acrylamide gels and Fig. 5B shows the pattern obtained at 12, 18, 30, 36, 48 and 72 h after infection compared to that obtained with type 5 infected cells. It can be seen that by 72 h post-infection polypeptide synthesis is almost entirely shut off.

Examination of the labelling patterns shown in Fig. 5 and of the patterns from other similar gels has indicated that there are consistent differences in the polypeptides produced by the two virus types and these differences are shown schematically in Fig. 6 together with a suggested nomenclature for some of these polypeptides. Considering these polypeptides in order of increasing electrophoretic mobility, it is clear that there is a difference in the mobilities of the hexon polypeptides of the two types (as in Fig. 5B) corresponding to a mol. wt. of about 100,000 for the type 12 hexon polypeptide in contrast to a value of 115,000 for type 5 (Russell & Skehel, 1972). A difference can also be seen in the mobilities of the ICSP-1 polypeptides although the ICSP-2 species appear to have similar mobilities. Surprisingly, in
Polypeptides of adenovirus type 12

Fig. 5. (A) Autoradiograms after electrophoresis of human embryo kidney cell extracts in 10% polyacrylamide gels. Cells were labelled with [35S]-methionine for 1 h at the indicated times after infection with type 12 virus. The pattern from uninfected cells labelled at 24 h after mock infection (C) and of a reference [35S]-methionine labelled type 5 virus (SV) is also shown. The positions of the hexon (H), penton base (PB) fibre (F) and core polypeptides (C-1 and -2) of the type 5 virus are also marked.

View of the polypeptide pattern noted with the complete particles there is a polypeptide of similar electrophoretic mobility to the type 5 penton base seen until 24 h after infection with type 12 virus (Fig. 5A). At later times this cannot be observed and it therefore seems that either this polypeptide is not made or is made and degraded and it may be significant that at these times a polypeptide can be seen which has a mobility similar to the presumed penton base polypeptide seen in the complete particle. The fibre polypeptide is relatively
Fig. 5. (B) Autoradiograms after electrophoresis of human embryo kidney cell extracts in 7.5% polyacrylamide gels. Cells were labelled with [35S]-methionine for 1 h at the indicated times after infection with either type 5 or type 12 virus. The patterns from uninfected cells labelled at 24 h after mock infection (C) and of a reference type 5 virus (5V) are also shown. The position of the structural polypeptides of type 5 virus are indicated as in (A) above. The extracts with c superscripts had been infected in the presence of 25 μg/ml of cytosine arabinoside. X indicates the major labelled polypeptide of the uninfected cells. In both (A) and (B) the origins of the gels are at the bottom of the autoradiograms.
Fig. 6. Schematic diagram of infected cell polypeptides, in cells infected with types 12 and 5 showing relative mobilities. On the right of the figure are the ICSP's already described for type 5 infected cells (Russell & Skehel, 1972, Fig. 2) while in the centre are the structural polypeptides characterized for type 5 virus. On the left of the figure are the differences noted in the polypeptides of type 12 infected cells.

poorly labelled in the type 12 infected cells and it is difficult to distinguish from the more heavily labelled, slightly more mobile, ICSP-3 (previously designated I-3 in Fig. 5A). The rationale for the assignment of the ICSP-3 in the type 12 infected cells can be explained by reference to Fig. 5B where the effect of cytosine arabinoside on the pattern of polypeptide synthesis in infected cells is shown. Previous experiments with type 5 infected cells demonstrated that, in the presence of this inhibitor only the production of the ‘early’ ICSP-3 polypeptide of mol. wt. 65,000 could be detected (Russell & Skehel, 1972). In the experiment shown here with the inhibited type 5 infected cells there is also a ‘leak’ of some of the later structural and ICS polypeptides although the ICSP-3 is the most prominently labelled polypeptide. However, in type 12 infected cells in the presence of the cytosine arabinoside
the major polypeptide has a mobility just slightly faster than the fibre polypeptide. This same polypeptide is apparently the one that was noted early in the uninhibited infection before the appearance of the capsid polypeptides, and is similar to the ICSP-3 polypeptide seen in type 5 infection in this respect also. Since in type 12 infected cells there was no indication of a polypeptide of similar mobility to the ICSP-3 seen in type 5 infection this polypeptide is therefore designated ICSP-3 although it has a significantly greater mobility than the corresponding type 5 polypeptide. It is interesting that the adenovirus T antigen can be produced under similar conditions (Gilead & Ginsberg, 1965) and it may be that ICSP-3 is a component of this antigen. Since the electrophoretic mobilities in this SDS electrophoresis system appear to be directly related to the mol. wts. of the polypeptides (Shapiro, Vinuela & Maizel, 1967) it can be estimated (using the other polypeptides as references) that this polypeptide has a mol. wt. of approximately 55,000.

Since there are polypeptides in the type 12 infected cells of similar mobilities to the ICSP's 4 and 5 of the type 5 infected cells it seems reasonable to assign these polypeptides similar ICSP numbers. This procedure, however, creates some difficulty in naming these other polypeptides noted in the type 12 infected cells which have mobilities between ICSP's 4 and 5. In particular, two of these polypeptides (designated 4A and 4B in Fig. 6) are quite prominent.
Polypeptides of adenovirus type 12

Fig. 8. Densitometer tracings of autoradiograms after electrophoresis of uninfected cells and of adenovirus 12 infected cells, labelled with $[^{32}P]$-orthophosphate from 16 to 48 h after infection. The tracings from $[^{32}P]$-labelled adenovirus type 12 virus and $[^{35}S]$-methionine labelled adenovirus type 5 virus are also shown. Intense labelling at the origin of gels (at the left) reflects retained $[^{32}P]$-labelled nucleic acids. Arrows indicate the major differences in the $[^{32}P]$-labelling patterns of the infected and uninfected cells.

infection and it can be seen that their relative stoichiometry is quite different early and late in infection. This is shown in Fig. 7 where densitometer tracings of autoradiograms obtained from cells pulsed at various times after infection are shown. Reference to this Fig. and Figs. 4 and 5 will show that at least some of the polypeptides, $\alpha$, $\beta$, and $\gamma$ found associated with the incomplete particles could be ICSP polypeptides, e.g. polypeptide $\alpha$ has a mobility similar to ICSP-4 and polypeptide $\beta$ is similar to either ICSP 4A or B. It is also evident on the other hand that polypeptide $\gamma$ has a similar mobility to a polypeptide in the uninfected cells and which becomes relatively prominent in infection (e.g. in the 12, 18 and 24 h samples in Fig. 5A). Fig. 7 also shows that ICSP-3 synthesis continues until relatively late in infection in contrast to the situation in type 5 infection where ICSP-3 synthesis is inhibited at later times. It may also be significant that cellular polypeptide synthesis (as demonstrated by synthesis of polypeptide X in Figs. 5A and 7) is similarly not inhibited to the same extent.
as in type 5 infection (Russell & Skehel, 1972). Examination of Fig. 7 shows that all polypeptides appear to be synthesized independently of one another and it is not evident whether some of them may be cleavage products of the others. These experiments also show that Y is a ‘late’ polypeptide, its synthesis being detected at 24 h concurrently with the capsid polypeptides.

Phosphorylation of infected cell polypeptides

Previous investigations have shown that on labelling cells infected with type 5 virus with high specific activity \(^{32}\)P-orthophosphate, three phosphorylated polypeptides can be detected by SDS polyacrylamide gel electrophoresis and autoradiography (Russell et al. 1972). A somewhat similar pattern of phosphorylation can be seen in type 12 infected cells (Fig. 8), the major difference from the uninfected cell pattern being a peak corresponding in mobility to the fibre polypeptide. It is noteworthy, however, that the intensity of labelling of the fibre is apparently less in the type 12 infected cells than in the type 5 infection. This finding probably reflects the relatively poor synthesis of fibre polypeptide in these cells which was noted above. Fig. 8 also indicates that type 12 purified \(^{32}\)P-labelled virus contains phosphorylated fibre polypeptide. In contrast to type 5, however, there is also some evidence of phosphorylation of the core-1 polypeptide.

DISCUSSION

Previous investigators (Maizel, White & Scharff, 1968a, b) using SDS polyacrylamide gel electrophoresis have shown that the structural polypeptides of types 2 and 12 adenovirus, while being broadly similar, did show significant differences which, however, were not further detailed. Analysis of the ‘top’ components of these viruses by the same workers also showed that they were deficient in core polypeptides. The studies described in this investigation have further delineated the differences between the polypeptides of the virus particles of types 5 and 12 and also the polypeptides of the cells infected by the two types.

DNA-DNA sequence homology studies (Green, 1970) and other techniques have shown a close similarity between types 2 and 5; however, both of these types, although they all share a common group specific antigen, have only about 20% of nucleotide sequences in common with the type 12 virus (Green, 1970). It is not, therefore, altogether surprising that significant differences can be seen in the electrophoretic mobilities of the structural polypeptides of types 5 and 12 and in the polypeptides produced in the infected cell. On the other hand, the similarities in both the stoichiometry and mobilities of some of the polypeptides of the two virus types allows one presumptively to assign bands to the hexon, fibre and two core polypeptides of type 12 virus without isolation of the individual proteins concerned.

If one assumes that the differences in mobilities seen in electrophoresis are related to differences in mol. wts. of the polypeptides (Shapiro et al. 1967) then there appears to be a difference in mol. wt. of the hexon polypeptides of types 5 and 12 of about 15000. This fairly large discrepancy in mol. wt. does not seem to be reflected in the size of the complete virus particles (Norrby & Ankerst, 1969; M. V. Nermut unpublished observations). This difference could be the result of the polypeptides assuming different conformations in the two types or that some smaller peptides may be associated with the type 12 polypeptide and are not detected on electrophoresis. The inability to assign conclusively a particular polypeptide to the penton base of type 12 virus may be a reflection of the fragility of this component. Thus, a previous report (Norrby & Ankerst, 1969) had noted that the pentons of type 12 were more fragile than other types, and it is also known that with types 2 and 5 the penton base is particularly susceptible to proteolysis (Pereira & Skehel, 1971). One could speculate, that the penton
Polypeptides of adenovirus type 12

base polypeptide being perhaps very susceptible to proteolytic enzymes is heavily nicked during the later intracellular assembly processes and although biologically and structurally functional, on denaturation with SDS, mercaptoethanol and urea, releases many small fragments which are not detected on electrophoresis. The significance of polypeptide Y which is seen in type 12 virus particles and not in type 5 virus is not clear. This polypeptide on the evidence presented here does not appear to be a component of either the hexon, penton base or fibre structural units and thus may be either packed inside the virus particle or in some way packed between the capsomeres. It may be of interest that the genome of the type 12 virus is significantly smaller than that of type 5 (Green et al. 1967) and thus there may be more space to allow packaging of an extra polypeptide.

No attempts were made in these experiments to characterize the incomplete particles in more detail. Smith (1965) showed that a number of different populations of incomplete type 12 particles could be separated. These populations had differing densities and morphologies ranging from apparently complete to ‘ragged-looking’ particles. Shimojo et al. (1967) were able to separate two populations one of complete particles of density 1.34 and the other of incomplete particles of density 1.29 to 1.31. Mak (1971) also demonstrated that two populations differing in density by 0.003 g/ml could be separated from the complete particle population and these had differing biological properties. Other investigators have examined types 2, 3 and 16 incomplete virus particles. Wadell, Hammarskjold & Varsanyi (1973) were able to separate a number of populations of incomplete particles of type 16 of differing densities while Sundquist et al. (1973) separated two populations of incomplete particles of type 3 virus. In both these investigations, moreover, it was noted that the incomplete particles contained polypeptides not present in the complete particle. Thus, it is possible that the complete and incomplete populations of type 12 although they were well separated in our experiments each consisted of a number of different kinds of particles. It cannot be ruled out also that the incomplete particles contain some DNA either virus or cellular. Nevertheless, it is quite clear that the polypeptide pattern was quite different and reproducible in each of the two types of particles. In the incomplete particles there were no core polypeptides but there were other polypeptides (\(\alpha\), \(\beta\) and \(\gamma\)) not noted in the complete particles, in agreement with both of the above investigations. The origin of these polypeptides cannot as yet be determined although in mobility they are similar to both infected cell specific and uninfected cell polypeptides. It seems possible, from their electrophoretic mobilities and also from the fact that their relative stoichiometries seem to vary that some of the polypeptides \(\alpha\), \(\beta\), \(\gamma\) and Y may be cleaved to form the core polypeptides and/or may be involved in some way in the assembly of the virus perhaps in a similar fashion to that described for poliovirus (Summers & Maizel, 1968; Jacobson, Asso & Baltimore, 1970). The results of Sundquist et al. (1973), suggesting that incomplete particles are precursors of complete particles, lend some credence to this proposition.

A finding of considerable interest in the adeno 12 infected cells was that early in infection a polypeptide (designated ICSP-3) of mol. wt. about 55,000 could be discerned. This polypeptide was also an ‘early’ component in that it appeared in the absence of DNA synthesis and thus probably corresponded to the previously described ‘T’ antigen. Several attempts have been made to demonstrate this more directly by immunoprecipitation with tumour sera but these have been unsuccessful probably because of the relatively poor potency of the tumour sera which were available. The properties of the T antigen have been extensively investigated and up to four mol. wt. species varying in mol. wt. from 2 to 15 \(\times\) 10^4 have been found associated with this antigen (Tockstein et al. 1968; Hollinshead et al. 1969; Potter, Oxford & McLaughlin, 1970). The nature and function of this antigen in infection and
oncogenesis is not apparent, although it is of interest that the corresponding polypeptide in the nononcogenic adenovirus type 5 infected cells is significantly higher in mol. wt.

The other significant feature of the polypeptides seen in type 12 infected cells when compared to type 5 infection was the presence of other polypeptides seen in the electropherogram between the core-1 and core-2 polypeptides. Whether these polypeptides are related to the polypeptides seen in the incomplete particles is still to be determined. One other interesting factor is the relative prominence of the ICSP-3 polypeptide even late in infection — in type 5 infected cells, the corresponding polypeptide cannot be detected later in infection and only the capsid polypeptides can be seen (Russell & Skehel, 1972). It is also apparent from an examination of the densitometer tracings that the cut-off of cellular polypeptide synthesis is much slower in type 12 infection and it may be that all these factors are related. Thus, the polypeptides noted between the core-1 and core-2 polypeptides may be cellular polypeptides which for some reason are under different regulatory controls than other cellular polypeptides. The relation of these findings to the oncogenic potential of the type 12 virus is obviously still a matter for speculation. It was also noted in type 12 infected cells that the synthesis of fibre and penton base polypeptides appeared to be much less than in type 5 infected cells and this is presumably a real difference since the intensity of the fibre bands in the purified viruses seem to be comparable. (On the other hand, it should be borne in mind that the differences noted above for the intensity of the ICSP-3's between the various types could reflect a different methionine content.)

The finding of phosphorylated polypeptides with type 12 virus as well as with type 5 infection suggests that this pattern of phosphorylation may be characteristic of adenovirus infection. In both types, the fibre polypeptide was significantly phosphorylated but there was no evidence of phosphorylation of core-1 polypeptide in type 5 as noted in type 12 infection. It will be of some interest to determine if the phosphorylated polypeptides play a significant role in the infecting process or if the phosphorylation reflects acceptor activity of the polypeptides (Tao & Doerfler, 1972) at later stages of the replication cycle.

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