Polypeptide Phosphorylation in Adenovirus-Infected Cells

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SUMMARY

Cells infected with human adenovirus type 5 have been labelled with $^{32}$P-orthophosphate under various conditions and extracts examined, after denaturation in sodium dodecyl sulphate (SDS), by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. A number of polypeptides appear to be phosphorylated specifically as a result of infection. Early in infection, phosphorylation of a polypeptide of apparent mol. wt. 26 K associated with ribosomes can be detected. Two other phosphorylated polypeptides of apparent mol. wt. 72 K and 18 K can also be seen, the former being mainly confined to the nucleus and capable of being precipitated by the previously described virus-specific P antiserum. The 18 K phosphorylated polypeptide is found mainly in association with membrane fractions. Later in infection phosphorylated polypeptides of apparent mol. wt. 100 K and 39 K can be recognized, the former being associated with ribosomes but removed, however, with a high salt wash; the latter component is mainly detected in the nucleus. Analysis of the purified $^{32}$P-labelled virus by the SDS PAGE technique indicated that a structural polypeptide of apparent mol. wt. 66 K (IIIa) was also phosphorylated.

INTRODUCTION

During infection with adenoviruses there are clearly a number of regulatory events occurring at several different levels. Thus, analyses of temperature-sensitive mutants of the viruses have shown that there is more than one gene controlling virus DNA synthesis; other events in the infected cell such as inhibition of cellular macromolecular synthesis, transport of virus polypeptides and assembly of the virus can also be related to the expression of a number of virus genes (Russell, Newman & Williams, 1972a; Russell, Skehel & Williams, 1974; Williams, Young & Austin, 1974). The nature of these regulatory processes is little understood and one possibility that we have been investigating for some time is that phosphorylation of polypeptides during infection could provide a means of modifying these polypeptides sufficiently to account for some of the events occurring during virus infection. Our earlier studies on phosphorylation of polypeptides during infection with adenovirus type 5 (Russell et al. 1972b) indicated that there were at least three polypeptides phosphorylated during infection, one of these being a structural component. We have expanded these investigations using more refined techniques of electrophoresis and cell fractionation and have been able to demonstrate that there are possibly five or more significant phosphorylations occurring during infection.
METHODS

**Virus and cells.** Adenovirus type 5 (Ad 75) was propagated in KB cells growing either as monolayers or in suspension. Virus seeds were prepared and titrated as described previously (Russell et al. 1967). The temperature-sensitive mutant ts 36 was used as detailed in previous publications (Russell et al. 1972a; Russell et al. 1974). Experiments involving analyses of labelled polypeptides were carried out on infected monolayers of HeLa or HEK cells (a continuous line of human embryo kidney cells) growing in Eagle’s medium (Russell & Skehel, 1972).

**Purification and labelling of virus.** Infected cells were labelled with $^{32}$P-orthophosphate or with $^{35}$S-methionine 6 to 8 h p.i. as described previously (Russell et al. 1972b). Virus was purified from the labelled infected cells by extraction with fluorocarbon followed by a velocity and two equilibrium centrifugations in caesium chloride (Russell, Laver & Sander- son, 1968; Winters & Russell, 1971). Virus preparations had approx. $1 \times 10^6$ cts/min/mg/ml.

**General plan of labelling experiments.** HeLa or HEK cells growing as monolayers in 1 oz bottles were infected with virus at a multiplicity of approx. 20 p.f.u./cell in a small volume of medium (no serum) at 38.5 °C. After an adsorption period of 2 to 3 h, the cell monolayers were supplemented with fresh medium (no serum) to a volume of 5 ml and incubated further at 38.5 °C. The cells were then labelled at various times after infection by removing the medium and replacing with 0.5 ml of warmed medium deprived of either phosphate or methionine as appropriate and containing 5 μCi/ml of $^{32}$P-orthophosphate or 20 μCi/ml of $^{35}$S-methionine respectively (isotopes were of high specific activity and obtained from the Radiochemical Centre, Amersham, Bucks.). Labelling of cells was carried out for 1 h at 38.5 °C when the medium was decanted and the cells either shaken or scraped off into 5 ml of phosphate buffered saline. Centrifugation (500 g for 5 min) of this cell suspension was followed by resuspension of the cell pellet in 100 μl of hypotonic buffer solution (5 mm-tris/HCl, pH 7.5) and storage at −20 °C until analysed. In some experiments the labelled cells were 'chased' by removing the radioactive medium, washing the cell sheet with warm complete medium and continuing the incubation at 38.5 °C in complete medium. In one series of experiments the cell pellets were directly resuspended in sodium dodecyl sulphate (SDS)/urea/mercaptoethanol (2%/5 m/3 m), namely SDS denaturing solution, prior to polyacrylamide gel electrophoresis.

**Cell fractionation procedures.** Investigations involving subcellular fractionations were carried out on samples of about $30 \times 10^6$ labelled cells obtained from 2 to 3 twenty oz bottles of HeLa or HEK cell monolayers. Volumes of infecting virus and labelled medium were increased appropriately. All operations were performed at 4 °C. Cell monolayers were removed by scraping into tris buffered saline solution (35 mm-tris/HCl, pH 7.5, 146 mm-NaCl) followed by washing the cells three times by centrifuging (500 g for 5 min) in 50 ml portions of the same buffer. During the final washing, 5 ml samples of the resuspended cells were removed, centrifuged and resuspended in 200 μl of 5 mm-tris/HCl, pH 7.5, and retained for analysis of labelled total cell polypeptides. The final cell pellet was resuspended in 4 ml 10 mm-tris/HCl, pH 7.5, 1.5 mm-MgCl₂, 15 mm-KCl, 6 mm-2-mercaptoethanol and, after leaving 10 min on ice, was then homogenized with 15 strokes of an all-glass homogenizer. Tonicity was restored by the addition of 0.4 ml 200 mm-tris/HCl (pH 7.5), 50 mm-MgCl₂, 800 mm-KCl, 60 mm-2-mercaptoethanol. This suspension was then centrifuged (600 g for 5 min) yielding a pellet of crude nuclei and a crude cell supernatant extract. The nuclei were further purified by resuspension in 0.5 ml 20 mm-tris/HCl, pH 7.5, 0.25% (w/v) Triton
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X-100, 6 mm-2-mercaptoethanol and centrifugation through a 4 ml sucrose cushion (20 mm-tris/HCl, pH 7.5, 60% (w/v) sucrose, 6 mm-2-mercaptoethanol; 75000 g for 60 min). The nuclear pellet was then washed and resuspended in 200 µl 5 mm-tris/HCl, pH 7.5, the supernatant fraction being discarded.

The crude cell supernatant extract was centrifuged (10000 g for 10 min) and the pellet was washed and resuspended in 200 µl of 5 mm-tris/HCl, pH 7-6. This fraction might be expected to contain cell membranes and mitochondria and is referred to as the '10000 g pellet'. The supernatant fraction from the latter centrifugation was then further centrifuged (150000 g for 3 h) and the ribosomal pellet thus obtained was washed and resuspended in 200 µl of 5 mm-tris/HCl, pH 7-5. The supernatant fraction obtained is referred to as the 'cytoplasmic extract' and was dialysed against 500 vol. of distilled water for 15 h, lyophilized and resuspended in 200 µl of 5 mm-tris/HCl, pH 7-5.

The ribosome preparation was further extracted by incubating for 1 h at 4°C in a hypertonic buffer containing 20 mm-tris/HCl, pH 7-5, 500 mm-KCl, 6 mm-2-mercaptoethanol in a total volume of 500 µl. The extract was then re-centrifuged (150000 g for 3 h) and the supernatant fraction obtained (referred to as 'ribosomal wash') was further dialysed against 500 vol. of distilled water for 15 h, lyophilized and resuspended in 50 µl of SDS denaturing solution prior to electrophoresis. The ribosomal pellets (termed 'washed ribosomes') were washed with a small volume of 5 mm-tris/HCl, pH 7-6 buffer and resuspended in 50 µl of SDS denaturing solution for electrophoresis.

**Polyacrylamide gel electrophoresis (PAGE) and autoradiography.** Cell extracts in 5 mm-tris/HCl buffer, pH 7-6, were sonicated for about 10 s (in bath-type sonicator: Megason) and samples were denatured by heating at 100°C for 2 min in 1% SDS, 1 mM-mercaptoethanol, 3 M-urea and then placed in the slots of a 5% spacer polyacrylamide slab gel with a resolving gel of 16% polyacrylamide of total slab length 150 mm and electrophoresis carried out using a discontinuous SDS system (Laemmli, 1970; Anderson, Baum & Gesteland, 1973) employing tris-glycine SDS electrophoresis buffer of pH 8.4. The electrophoresis was normally completed after an overnight run at 70 V and 20 mA. The gels were washed in a destaining fluid of acetic acid:methanol:water (2:5:10) for about 2 to 3 h and then dried down under vacuum on a porous polypropylene filter. In some cases the gels were stained with 0.1% Coomassie blue in the acetic acid/methanol/water solution for 30 min at room temperature before washing in the destaining fluid. The dried slabs were then exposed to X-ray film for the appropriate time (normally 18 to 48 h) and developed using standard procedures. Densitometer tracings were obtained with the Joyce–Loebl integrating microdensitometer.

**Antisera** to P. antigen were prepared in rabbits as described previously (Russell et al. 1967).

**Immunoprecipitation** was carried out using a modification of the method described by Horvitz (1974). Cell pellets were suspended in buffer A (10 mm-tris/HCl, pH 7-9, 10 mm-MgCl₂, 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 5% glycerol) and disrupted by sonication. 150 µl of this suspension was made 0.5 M with respect to KCl by adding 5 M-KCl and then incubating overnight at 4°C with RNase (150 µg/ml) and DNase (100 µg/ml). The digestes were then centrifuged (10000 g for 30 min) and the supernatant fluids reacted with the appropriate antisera. This was carried out by adding to 50 µl of the supernatant fluid, 70 µl of TTK buffer (0.1 mM-tris/HCl, pH 7-8, 2% Triton X-100, 2 M-KCl) in the presence of 25 µl of rabbit antiserum (or a dilution in phosphate buffered saline) and incubating at 4°C overnight. Forty µl of goat anti-rabbit globulin was added and the mixture incubated at room temperature for 30 min and then centrifuged (10000 g for 30 min). The supernatant
Fig. 1. Structural polypeptides of adenovirus type 5. (a) Coomassie blue stained electropherogram after SDS PAGE analysis of purified virus (origin at top of figure); (b) $^{32}$P-labelled purified virus (prepared as in Methods) – stained electropherogram and corresponding autoradiogram. (Staining at origin of autoradiogram is from labelled DNA.)

fluids were removed and the pellets taken up in 50 µl of denaturing SDS solution prior to electrophoretic analysis.

Nomenclature. The terminology proposed by Ginsberg et al. (1966) for the major capsid components (hexon, penton and fibre) and by Russell, McIntosh & Skehel (1970) for the internal components (core-1, core-2 proteins) is utilized. Other structural components and polypeptides are referred to using the terminology proposed by Maizel, White & Scharff (1968) and Anderson et al. (1973) for adenovirus type 2.

RESULTS

Structural polypeptides

Previous investigations using the continuous SDS PAGE system (Russell et al. 1971; Russell & Skehel, 1972) have shown that purified adenovirus type 5 contains at least five major polypeptides detected both by direct staining of the gel and by autoradiography of $^{35}$S-labelled virus. Using the discontinuous SDS PAGE system as described in Methods it became evident that with its improved resolution, particularly in the smaller mol. wt. region, other polypeptides not previously recognized could be detected. Fig. 1 (a) shows a Coomassie stained electropherogram of purified adenovirus type 5 together with the nomenclature previously proposed (in centre) and the nomenclature used by Maizel et al. (1968) and Ande-
son et al. (1973) for adenovirus type 2. At least 11 components can be clearly detected in this electropherogram and these polypeptides were consistently seen in many analyses, both by direct staining and by autoradiography of purified $^{35}$S-methionine-labelled virus. Better resolution of the smaller mol. wt. region can be obtained by using gels with a higher urea content and three components (X, XI and XII) can be recognized making 13 polypeptides in all (D. Rekosh, personal communication). It is particularly noticeable that the fibre polypeptide can be clearly separated from another polypeptide (IIa) with a slightly lower electrophoretic mobility. The assignment of the fibre to the faster component was made (data not shown) by electrophoresis of a sample of purified crystalline fibre (Mautner & Pereira, 1971) in parallel with a virus preparation. (Only very rarely was the fibre polypeptide separated from the IIa polypeptide when using the continuous SDS PAGE system.)

On analysing purified $^{32}$P-labelled virus by the discontinuous SDS PAGE technique, autoradiography indicated the presence of one major labelled component, the electrophoretic mobility corresponding to the IIa polypeptide. Fig. 1(b) shows a Coomassie stained electropherogram and the corresponding autoradiogram of a $^{32}$P-labelled purified virus preparation. Longer exposures (i.e. 2 to 3 weeks) also indicated the presence of labelled polypeptides corresponding to polypeptides V (core-1), VI and IX, but these were apparently only minor modifications compared to that of the IIa component. Since previous experiments (Russell et al. 1972b) had demonstrated that the major phosphorylated structural component was sensitive to pronase and insensitive to RNase and DNase, these results suggested that this labelled component was the phosphorylated IIa polypeptide and not the fibre as originally proposed.

Infected cell polypeptides

In addition to the structural components described above, other polypeptides not present in the virion could be detected in cells infected with adenovirus type 5 by labelling with $^{35}$S-methionine followed by SDS PAGE analysis and autoradiography. Five of these components termed ICSPs (infected cell specific polypeptides) had been detected using continuous SDS PAGE (Russell & Skehel, 1972), whereas the discontinuous system resolved at least eight non-structural polypeptides associated with infection by adenovirus type 2 (Anderson et al. 1973; Walter & Maizel, 1974). Fig. 2 illustrates the pattern of labelling with $^{35}$S-methionine seen in uninfected and adenovirus 5 infected cells using the discontinuous SDS PAGE technique and it will be seen that at least eleven polypeptides can be recognized in the infected cell which have different labelling characteristics than those seen in uninfected cells, and differ in electrophoretic mobility from the structural polypeptides. Some of these polypeptides have been shown with adenovirus type 2 infected cells to be precursors to structural components (Anderson et al. 1973; Ishibashi & Maizel, 1974; Öberg et al. 1975) and similar results have been obtained in cells infected with type 5 virus (unpublished data). The terminology used to describe these polypeptides has differed in the various laboratories studying polypeptide synthesis in adenovirus infected cells. On the extreme right of Fig. 2, we have used the terminology proposed by Anderson et al. (1973) for adenovirus type 2 infected cells. This procedure depends on an assignment of mol. wt. to a given polypeptide and this in turn is, to some extent, a function of the standards used to determine mol. wt. Furthermore, although adenovirus type 2 and 5 are very similar antigenically and in their DNA sequence homology a surprising number of differences in electrophoretic mobility between their polypeptides have been shown (T. Grodzicker, C.W. Anderson & J. Sambrook, personal communication). Thus, although our values for mol. wt. of these polypeptides in type 5 infected cells do differ (unpublished observations) from those described by others for
type 2 infected cells, in the interests of clarity we have, as far as possible, retained the same nomenclature. The analysis shown in Fig. 2 does not resolve polypeptides of mol. wt. below about 10000, and therefore does not detect smaller polypeptides synthesized, or processed, during infection. It should be noted that some of the structural polypeptides (namely VI and VIII) cannot be detected in this particular pattern, and that there are other polypeptides (39 K and 24 K) not previously described.

Fig. 2 also shows the pattern of labelling seen with $^{32}$P-orthophosphate in uninfected and adenovirus type 5 infected cells. Those phosphorylated polypeptides which appear to be specific for the virus-infected cell are listed on the left, the numbers assigned being their presumed mol. wt. relative to $^{35}$S-methionine-labelled polypeptides. In other experiments the major phosphorylated component, 39 K, can be resolved into two bands. All of these phosphorylated components are similar in electrophoretic mobility to $^{35}$S-methionine-labelled polypeptides described in the infected cell but this, of course, does not imply that they are necessarily identical. This pattern of $^{32}$P labelling was consistently obtained in a large number of experiments and the pattern did not seem to depend on the method of extraction of the infected cells or whether the cells were lysed directly into SDS denaturation
solution, i.e. the labelling did not seem to arise from \textit{in vitro} labelling of the extracts with endogenous protein kinases.

The pattern of $^{32}$P labelling seen in cells infected with \textit{ts} 36 (an 'early' mutant) at 38.5 °C as shown in Fig. 2 is similar to that found in cells infected with virus in the presence of an inhibitor of DNA synthesis such as cytosine arabinoside (data not shown) and indicates the 'early' pattern, i.e. prior to virus DNA synthesis. Previous experiments (Russell & Skehel, 1972; Bablanian & Russell, 1974; Russell, 1974) have demonstrated that the $^{35}$S-labelled 72 K protein (ICSP-3) is the major early protein and presumably the phosphorylated component reflects phosphorylation of the same protein. Since the $^{35}$S-methionine-labelled 100 K polypeptide appears 'late' in infection (Bablanian & Russell, 1974) and the corresponding phosphorylated derivative does not appear in the absence of virus DNA synthesis this suggests that the phosphorylation in this case also reflects modification of the same 100 K polypeptide. In contrast, the 26 K $^{35}$S-labelled polypeptide seen in infected cells appears to be synthesized late in infection whereas the phosphorylated component appears early in infection. Indeed, closer inspection of many gels reveal that the phosphorylated component has a slightly lower mobility than the $^{35}$S-methionine-labelled polypeptide of similar mol. wt. and thus, in this case, the $^{32}$P-labelled 26 K may reflect modification of a minor virus polypeptide or of a cellular component. Changes in the pattern of phosphorylation of components of lower mol. wt. can be seen by exposing the electropherograms (e.g. as used for Fig. 2) to photographic film for a longer time. These differences are illustrated in

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Fig. 4. $^{32}$P labelling patterns at different times after infection. (a) HEK cells were labelled with $^{32}$P-orthophosphate for 1 h at 4 h (4 V) and 12 h (12 V) p.i. at 38.5 °C. Cells were also mock-infected and labelled at 12 h (C). Autoradiograms were derived from these extracts after SDS PAGE analysis; (b) HeLa cells were labelled for 1 h at 8 h (8 V), 12 h (12 V) and 24 h (24 V) p.i. at 38.5 °C with $^{32}$P-orthophosphate or $^{35}$S-methionine. Cells were also mock-infected and labelled at 12 h (C). Autoradiograms were derived from the extracts of these cells after SDS PAGE analysis.

The densitometer tracings from another experiment in Fig. 3. It can be seen that the infected cells as well as showing increased incorporation of $^{32}$P label into the 26 K component also show incorporation of label into a component of apparent mol. wt. 18 K and the reduction of incorporation into components of slightly lower and higher electrophoretic mobility seen in labelled uninfected cells. In Fig. 2 and all other gels examined it is quite difficult to discern clearly a virus specific $^{32}$P-labelled polypeptide corresponding in mobility to the IIIa polypeptide (66 K) particularly since there is a labelled component seen in the same position in uninfected cells. We have already shown that this is the major phosphorylated component in the virus particle (Fig. 1) and therefore it is presumed that this phosphorylation event in terms of acceptance and retention of the $^{32}$P label under the labelling conditions is a relatively minor event.

Fig. 2 also gives some indication that at least two of these polypeptides are phosphorylated early in infection (i.e. before virus DNA synthesis). Thus the 72 K and 26 K components can be clearly seen in the autoradiograms derived from cells infected with the $ts$ 36 mutant at the restrictive temperature. By pulse labelling infected cells with $^{32}$P-orthophosphate at different times after infection it was interesting to note as in Fig. 4(a) that phosphorylation of the 26 K component could be demonstrated readily quite early in infection (i.e. at 4 h) even before the 72 K phosphorylation was evident, suggesting that this phosphorylation may be...
amongst the earliest events in the infectious process. Fig. 4(b) also illustrates the results from a similar experiment in which cells infected in parallel were also labelled with $^{35}$S-methionine at different times after infection. This experiment shows that at later times when there was no synthesis of 72 K polypeptide there is substantial phosphorylation of the polypeptide occurring, suggesting that the modification is a post-synthetic event and can occur quite late in infection when synthesis of this polypeptide has been terminated.

A number of experiments were carried out to ascertain the sensitivity of the various
Fig. 6. 'Pulse-chase' of $^{32}$P-orthophosphate in adenovirus infected cells. HEK cells were labelled at 18 h p.i. (V) for 1 h (p) with $^{32}$P-orthophosphate or $^{35}$S-methionine. Mock infected cells (C) were similarly labelled. Replicate cultures were also labelled and then 'chased' with complete medium for a further 16 h (ch). Extracts were submitted to SDS PAGE analysis and the autoradiograms derived from the $^{35}$S-methionine labelled cells are shown on the left and $^{32}$P-orthophosphate labelled on the right.

$^{32}$P-labelled components to protease and ribonuclease digestion. Figure 5 illustrates one such experiment and indicates that all of the components already designated are sensitive to pronase and insensitive to RNase. Two components can be seen to be sensitive to RNase and insensitive to pronase – a component seen only in infected cells corresponding in mobility to a polypeptide of mol. wt. about 44 K, and a more diffuse component corresponding in mobility to a polypeptide of mol. wt. 19 to 20 K. This latter component is probably related to the VA and related RNAs (Ohe, Weissman & Cooke, 1969) and to transfer RNAs, since transfer RNAs will migrate to this position in these gels (data not shown).

In Fig. 6 patterns derived from cells which had been labelled for 1 h and then 'chased' for 16 h can also be seen. Under these conditions the phosphate label corresponding to the 100 K and IIIa polypeptides is removed while the 72 K associated label is retained. This presumably reflects substantial turnover of the phosphate attached to the 100 K and IIIa components. After the chase procedure, labelling can be seen both in the 39 K region and about 41 K, the latter component presumably being RNA because of its sensitivity to RNase.
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Fig. 7. Immunoprecipitation of a2P-labelled extracts. Autoradiograms obtained after SDS PAGE analysis of immunoprecipitates. HEK cells were labelled for 1 h at 18 h p.i. with a2P-orthophosphate and the extracts were then incubated with rabbit anti-P serum and processed as described in Methods. Extracts of a2P-labelled uninfected cells were similarly processed. A rabbit antiserum corresponding to the P antiserum but against vaccinia antigens (K) was used as a control. a5S-labelled purified virus was electrophoresed in parallel.

(Fig. 5). The relatively late appearance of the a5P label in the 39 K region suggests that this phosphorylation is of a different nature from those involved in the 100 K and IIIa phosphorylation.

Some of the a5S-labelled polypeptides show minor alterations in mobility during the chase and this may be related to qualitative and quantitative changes in phosphorylation.

Immunoprecipitation

Previous experiments (Russell & Skehel, 1972) have shown that the ICSP-3 (72 K) polypeptide labelled with a5S-methionine could be precipitated with the so-called P antiserum (Russell et al. 1967). This antiserum was prepared in rabbits against antigens made early in infection of rabbit cells with adenovirus type 5 and the precipitability thus indicated the virus specificity of the a5S-labelled 72 K polypeptide. A similar experiment was conducted.
Fig. 8. The distribution of 35S-methionine-labelled polypeptides in subcellular fractions prepared from uninfected and adenovirus-infected HeLa cells. HeLa cells were grown, infected, labelled with 35S-methionine and fractionated as described in Methods. Samples (10 μl) of (a) total cell extracts, (b) nuclei, (c) 10,000 g pellet, (d) ribosomes and (e) cytoplasmic extract were analysed by SDS PAGE. C = mock-infected HeLa cells, I = adenovirus-infected HeLa cells. The results shown with the cytoplasmic extract fraction were obtained in a separate electrophoresis.

with extracted 32P-labelled cells infected in the presence of cytosine arabinoside. Figure 7 indicates that the 32P-labelled 72 K polypeptide could also be demonstrated in the precipitate obtained from the infected cells by incubation with P antiserum. This result substantiates the suggestion that the phosphorylated 72 K component is virus coded and therefore probably related to the 35S-methionine-labelled polypeptide.

Cell fractionation

To ascertain the location within the cell of the various phosphorylated polypeptides, cell cultures which had been labelled in parallel with 35S-methionine and 32P-phosphate were harvested and submitted to standard fractionation procedures as outlined in Methods. Figure 8 illustrates the labelling pattern seen in fractions derived from the 35S-methionine-labelled cells. On analysing the distribution of labelled polypeptides of interest to this study it will be seen that the 100 K 35S-labelled polypeptide appears primarily in the ribosome fraction, although small amounts can be detected in the other fractions. On the other hand, the 72 K polypeptide resides primarily in the nucleus although a small amount can also be detected in the cytoplasmic extract.

As indicated above, phosphorylated 26 K polypeptide does not seem to correspond in mobility to either of the major 26 K and 27 K 35S-polypeptides seen in infected cells; more-
Fig. 9. The distribution of \( ^{32}P \)-orthophosphate-labelled polypeptides in subcellular fractions prepared from uninfected HeLa cells, and HeLa cells infected with adenovirus in the presence and absence of 75 \( \mu \text{g/ml} \) cytosine arabinoside. HeLa cells were grown, infected, labelled with \( ^{32}P \)-orthophosphate and fractionated as described in Methods. The fractions were taken up in 200 \( \mu \text{l} \) of 5 \( \text{mm} \)-tris/HCl buffer giving subcellular equivalent suspensions. (a) Samples (10 \( \mu \text{l} \)) of total cell extracts (i) and nuclei (ii) were analysed by SDS PAGE. (b) Similarly, 10 \( \mu l \) samples of the 10000 \( g \) pellet (i), ribosomes (ii) and cytoplasmic extract (iii) from the same cell fractionation were analysed in a separate electrophoresis. U = uninfected HeLa cells. E = HeLa cells infected with adenovirus in the presence of 75 \( \mu \text{g/ml} \) cytosine arabinoside. L = adenovirus-infected HeLa cells.

Over, these polypeptides are probably precursors of structural proteins (Öberg et al. 1975). Since even a minor \( ^{35}S \)-labelled polypeptide of similar electrophoretic mobility to the phosphorylated component could not be discerned in the labelled patterns examined, it is assumed that the polypeptide is not significantly labelled under these conditions. It was notable, however, that a \( ^{35}S \)-labelled 18 K component could be detected in the 10000 \( g \) pellet fraction.

Figure 9 shows the labelling pattern seen in fractions derived from \( ^{32}P \)-phosphate-labelled cells. In this experiment infected cells were labelled in the presence (E) and absence of cytosine arabinoside (L) to determine the distribution of label ‘early’ and ‘late’ in infection respectively (Russell et al. 1967; Russell & Skehel, 1972). It is evident that the 100 K \( ^{32}P \)-labelled component appears in the ribosome fraction and also in the cytoplasmic extract. On the other hand, the 72 K phosphorylated polypeptide resides in the nuclear fraction with some also evident in the cytoplasmic extract. Similarly the 39 K component also appears to be confined mainly to the nucleus. It is also very notable that the ribosome fraction as well as harbouring the 100 K also has the phosphorylated 26 K component derived from both ‘early’ and ‘late’ infected cell extracts and it is interesting that the ‘wash’ with a high ionic strength buffer removes the 100 K phosphorylated component while the 26 K component is retained on the ribosomes, indicating that this component is tightly associated with the ribosomes (Fig. 10).
Fig. 10. The effect of washing $^{32}$P-orthophosphate-labelled ribosomes with buffer containing 500 mM-KCl. $^{32}$P-orthophosphate-labelled ribosomes were prepared from uninfected HeLa cells, and HeLa cells infected with adenovirus in the presence and absence of 75 µg/ml cytosine arabinoside as described in Methods. Portions (100 µl) of each sample of ribosomes were submitted to a high KCl salt wash procedure (see Methods), and the salt wash (i) and washed ribosomes (ii) were analysed by SDS PAGE. U = uninfected HeLa cells, E = HeLa cells infected with adenovirus in the presence of 75 µg/ml cytosine arabinoside, L = adenovirus-infected HeLa cells.

**DISCUSSION**

The experiments described in this paper are essentially a description of phosphate labelling patterns seen during adenovirus infection. It is hoped that further investigations on these phosphorylated polypeptides will indicate whether the modifications detected have any particular significance with regard both to events in the infected cells, and to the function of the phosphorylated structural components. Our current studies are directed towards an elucidation of the nature of the phosphate bonds involved in the modifications, and concomitantly we are investigating the enzymes, presumably protein kinase(s) and phosphatase(s), controlling these modifications. Tao & Doerfler (1972) had shown previously that in
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of most of the major structural polypeptides, suggesting that the more specific modifications noted in this communication may be of some significance. Of particular note is the detection of the phosphorylated IIIa polypeptide in the purified virus. The location of this polypeptide in the virion is not accurately known although current indications are that it resides in the penton region (Everitt, Lutter & Philipson, 1975) and iodination studies carried out by ourselves on adenovirus type 5 (unpublished data) suggest that it is located internally. If this is indeed the case then it is difficult to conceive of the phosphorylation occurring as a result of contaminating kinases although it cannot be ruled out that they may be trapped within the virus particle during maturation. Other studies (Lewis et al. 1975) also indicate that IIIa and fibre are different polypeptides and therefore the separation between these two components on electrophoresis in polyacrylamide gels does not merely reflect modification of the fibre polypeptide and a resultant change in electrophoretic mobility.

It may be of some significance that the phosphorylations noted in the infected cell appear to involve mainly non-structural polypeptides and it is tempting to speculate that the modifications may be related to the function of these components within the infected cell. It was also noted that the IIIa intracellular phosphorylation was not easy to detect under the labelling conditions employed, and furthermore, the label was removed under 'chase' conditions indicating turnover during infection, perhaps once again a reflection of the function of this structural polypeptide.

It is clear from the fractionation experiments that most of the phosphorylated polypeptides could be found preferentially in particular cellular fractions. In the case of the 100 K polypeptide the 35S-labelled entity could be detected in the nucleus and cytoplasmic fractions as well as associated with ribosomes. The 32P-labelled polypeptide was found preferentially, however, associated with ribosomes and removed by a high salt wash. This distribution is consistent with the hypothesis that this polypeptide may play some role in a virus messenger ribonucleoprotein complex as advocated by Lindberg & Sundquist (1974). Whether the phosphorylation of the polypeptide is of significance with regard to events at the level of translation where there is evidence of discrimination between virus and cellular polypeptide synthesis late in infection, remains to be demonstrated. It is of some interest in this respect that Auerbach & Pederson (1975) have shown that phosphorylation of messenger ribonucleoproteins does occur in uninfected HeLa cells.

The 72 K polypeptide has been shown to bind to single-stranded DNA and appears to be implicated in virus DNA synthesis (Van der Vliet & Levine, 1973; Levine et al. 1974). The experiments described here have suggested that this component is the major phosphorylation event during infection and it therefore seems very likely that the modification of this polypeptide could be related to its function during infection. Since it seems to be phosphorylated in the absence of virus DNA synthesis (i.e. when infected in the presence of cytosine arabinoside or when infected with ts 36 at the restrictive temperature) it may not be directly involved in virus DNA synthesis. On the other hand, it could be that the polypeptide has different sites available for phosphorylation at different stages of the replication process or alternatively it could have some function in relation to cellular DNA synthesis, since this is inhibited early in infection (Mäntyjärvi & Russell, 1969). It was also found that phosphorylation of this polypeptide occurred quite late in infection long after synthesis had ceased, suggesting that phosphorylation may be a continuous event in the infected cell and is independent of polypeptide synthesis. Our current studies are concerned with the relationship of the phosphorylation to the DNA binding ability of the polypeptide.

The phosphorylated 39 K polypeptide appears to be similar to the 72 K polypeptide in vitro phosphorylation using a partially purified reticulocyte kinase led to phosphorylation
its nuclear location but since it is evident only late in infection and can appear under 'chase' conditions it is probably not directly related to the 72 K polypeptide in its function. It is of some significance that the $^{35}$S-labelled 39 K polypeptide which appears to have the same location as the $^{32}$P-labelled entity in the infected cell, appears as rather a diffuse band in autoradiograms being somewhat similar to the $^{35}$S-labelled 72 K polypeptide in this respect. This diffuse character of the $^{35}$S-labelled band may therefore be a function of different degrees of modification of the polypeptide. This polypeptide has not been recognized previously as being of any significance in infection and consequently nothing is known of its properties.

Since the phosphorylated 26 K polypeptide remains associated with ribosomes after the high ionic strength buffer wash it seems quite possible that this event could be ascribed to a modification of a ribosomal protein perhaps similar in nature to that discerned after infection by vaccinia virus (Kaerlein & Horak, 1976). In the latter case the major polypeptide involved (probably the S2 ribosomal protein) has a mol. wt. of about 41 K (Lin & Wool, 1974) and thus is different from that seen in the case of adenovirus infection. It is extremely interesting that the phosphorylation of this 26 K component is among the first events noted in infection and it is tempting to speculate that modification of cellular ribosomes may be of value in pre-empting the cellular machinery to facilitate virus replication.

The association of both the phosphorylated and methionine-labelled 18 K polypeptide with the pellet fraction suggests that this component may be an early phosphorylated virus polypeptide perhaps related to the small molecular weight glycosylated polypeptide described by Walter & Maizel (1974) in the membrane fraction of infected cells.

It will be evident that further studies will be required to assess the true significance of the phosphorylations described in this communication. Investigations in prokaryotic systems (Rahmsdorf et al. 1973; Horovitz, 1974) have shown that phosphorylation of host polypeptides are of some significance with respect to transcriptional and translational control during infection and have thus provided the precedent and the spur to further studies with eukaryotes.

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REFERENCES


Phosphorylated adenovirus polypeptides


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