The Polypeptides of Adenovirus-infected Cells

By W. C. RUSSELL AND J. J. SKEHEL

National Institute for Medical Research, Mill Hill, London NW7 1AA, England

(Accepted 9 December 1971)

SUMMARY

The polypeptides of cells which had been infected with adenovirus and pulse labelled with high specific activity $[^{35}\text{S}]$-methionine have been examined by polyacrylamide gel electrophoresis followed by autoradiography. Five virus-particle polypeptides and at least five other polypeptides which appeared to be specific for the infected cell could be discerned. One of the latter polypeptides could be detected very early in infection and is shown to be one of the major components of the previously described P antigen. The experiments also show that in the absence of arginine in the tissue culture medium, the infected cells fail to synthesize the arginine-rich core polypeptide.

INTRODUCTION

Several detailed investigations, using the technique of polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) have indicated that the adenovirus particle contains from five to nine polypeptide species which range in molecular weight from 10,000 to 120,000 and together account for approximately 35% of the polypeptide coding potential of the virus genome (Maizel, White & Scharff, 1968a, b; Laver, 1970; Russell, McIntosh & Skehel, 1971b; Russell, 1971). The synthesis of these virus components has also been detected in adenovirus-infected cells, particularly at the later stages of the infectious cycle when host protein synthesis has been depressed (White, Scharff & Maizel, 1969). It has, however, not so far been possible to detect virus-coded proteins which are not incorporated into particles. In this paper, in an attempt to characterize these components, we have labelled cells infected with adenovirus type 5 with high specific activity $[^{35}\text{S}]$-methionine and examined their polypeptide composition by SDS polyacrylamide gel electrophoresis and autoradiography.

METHODS

Virus and cells. Adenovirus type 5 (strain AD75) was propagated in KB cells growing in Leibovitz medium, using techniques described previously (Russell et al. 1967a; Russell, Valentine & Pereira, 1967b). Analysis of polypeptide synthesis during the infectious cycle was carried out using either a line of human embryo kidney cells (HEK), or a line of rabbit kidney cells (RK 13). These two cell lines were grown in Eagles medium containing the usual antibiotics, tryptose phosphate broth and 10% calf serum (Russell et al. 1967a).

Preparation of virus labelled with $[^{35}\text{S}]$-methionine. KB cells were infected in the manner described previously (Russell et al. 1967b). Approximately 8 hr after infection the tissue culture medium was removed and replaced with medium containing 5% of the normal amount of methionine (i.e. 4 µg./ml.) and $[^{35}\text{S}]$-methionine at 5 µC/ml. (specific activity
20 c/m-mole – obtained from the Radiochemical Centre, Amersham, Bucks). The infected cells were then incubated for a further 40 hr at 33° and the virus produced was purified by techniques described previously (Russell, Laver & Sanderson, 1968). Virus prepared in this way had a specific activity of about $1 \times 10^6$ counts/min./mg.

*Infection of cells and ‘pulse’ labelling with isotope.* In most experiments cells were infected in suspension at 37° by shaking for 30 min. with virus at an added m.o.i. of about 200. Samples of $1 \times 10^6$ cells were then dispensed into 1 oz. bottles and incubated at 38.5°, forming monolayers. No serum was added to the tissue-culture medium during infection. In some experiments cells were infected as monolayers in 1 oz. bottles by adding the virus in a volume of 0.5 ml. and then supplementing this with medium to a total of 4 ml., 3 to 4 hr later. In experiments in which cells were infected in the absence of arginine in the medium, the arginine depletion and infection procedures have been described previously (Russell & Becker, 1968).

Cells were pulse-labelled at various times after infection by removing the medium and replacing with 2 ml. of pre-warmed medium deprived of methionine but containing [35S]-methionine at 5 μc/ml. In experiments where a proportion of the infected cells had detached from the glass surface, the medium was centrifuged and the cell pellet was resuspended in the labelled medium and then the suspension added back to the cells remaining in the bottle. The labelling period was generally for 30 min. at 38.5°, following which the cells were cooled, scraped from the glass into the medium and centrifuged (500g, 5 min.). After washing by resuspension in 5 ml. of phosphate buffered saline, the cell pellet was finally resuspended in 100 μl. of 0.005 M-Tris + HCl buffer, pH 7.4, and stored at −70°.

*Polyacrylamide gel electrophoresis and autoradiography.* All extracts as prepared above were disrupted by ultrasonic vibration (Dawe Soniclean bath) and a suitable sample, normally 20 μl., containing about $1 \times 10^6$ counts/min. was dissociated by heating at 100° for 1 min. in the presence of 1% SDS, 2% mercaptoethanol and 8 M-urea in 1 μl-phosphate buffer, pH 7.2, and then applied to the top of polyacrylamide gel columns containing 7.5% (w/v) acrylamide, 0.2% SDS and 5 M-urea. Electrophoresis was carried out at 30 v and 3 mA tube for 14 hr (Pereira & Skehel, 1971). Samples of labelled virus were dissociated and electrophoresed in parallel.

After electrophoresis the gels were sliced longitudinally using an apparatus similar to that described by Fairbanks, Levinthal & Reeder (1965). The slices were washed twice in 7% acetic acid, laid on a porous filter, covered with cellophane and dried under vacuum for 16 hr. The dried and flattened strips, attached to the cellophane, could then be easily removed and submitted to autoradiography by standard techniques using X-ray film (Agfa–Gevaert, Osray M).

*Antisera* were prepared as described previously (Russell et al. 1967a). The hexon antiserum, prepared using crystalline hexon antigen (Pereira, Valentine & Russell, 1968), was kindly supplied by Dr H. G. Pereira. A vaccinia P antiserum was used as a control rabbit antiserum in one series of experiments. This serum was prepared in exactly the same way as the adenovirus P antiserum but using purified vaccinia virus.

*Fractionation of cells* into ‘nuclear’ and ‘cytoplasmic’ fractions using 0.5% Nonidet NP40 has been described previously (Mäntyjärvi & Russell, 1969).

*Immuno-precipitation.* Fifty μl. extracts of infected cells were centrifuged at 750g for 30 min. and then mixed with 50 μl. dilutions of antiserum and incubated at room temperature for 6 hr. These mixtures were then centrifuged at 750g for 30 min. and the supernatant fluids discarded. The pellets were dissociated directly with the SDS dissociating mixture and submitted to electrophoresis in the usual way.
Fluorocarbon extraction. Extracts of cells were shaken vigorously by hand with an equal volume of trichlorotrifluorethane (Arklone P, ICI Ltd) at room temperature for a few minutes. The aqueous and organic phases were separated by centrifugation (500g, 10 min.) and the aqueous upper layer and the viscous interphase were separately retained.

RESULTS

Radioactive polypeptides in adenovirus infected cells

Preliminary experiments were carried out using extracts of cells which had been either incubated continuously with $[^{35}S]$-methionine from 8 to 24 hr after infection or for only 30 min. at 10 hr after infection. The pattern of radioactive polypeptides obtained after electrophoresis and autoradiography of these infected cell extracts is shown in Fig. 1 together
Fig. 2. Schematic diagram of infected cell polypeptides, showing relative mobilities and suggested nomenclature.

**Table 1. Molecular weight estimates of the virus-particle and infected cell specific polypeptides**

<table>
<thead>
<tr>
<th>Virus particle polypeptides</th>
<th>ICSP's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexon</td>
<td>1</td>
</tr>
<tr>
<td>Penton Base</td>
<td>2</td>
</tr>
<tr>
<td>Fibre</td>
<td>3</td>
</tr>
<tr>
<td>Core 1</td>
<td>4</td>
</tr>
<tr>
<td>Core 2</td>
<td>5</td>
</tr>
</tbody>
</table>

The molecular weight estimates of the virus particle polypeptides are taken from Maizel et al. (1968a) and Russell et al. (1971b).
The polypeptides of adenovirus-infected cells

with that of extracts from uninfected cells and with that of labelled purified virus. It was
clear that five virus particle polypeptides (i.e. the three capsid polypeptides and two core
polypeptides) could be readily discerned in the infected cell extracts and moreover that other
radioactive polypeptides were apparent which were either absent from or relatively poorly
labelled in uninfected cells. It was also evident from the pattern of labelling that there was a
significant decrease in cellular polypeptide synthesis in the infected cells during the labelling
period. This pattern of polypeptides synthesized in infected cells was reproducible and more-
over was independent of the labelling period employed at least from 20 min. to 20 hr. The
technique thus provides a means of characterizing those other polypeptides which, while not
being components of the virus particles, appeared to be specific for the infected cell. As will
be shown below, these polypeptides in some cases have mobilities very similar to virus
particle polypeptides and are synthesized and shut off at different times to the virus
particle polypeptides. Since it cannot be unequivocally demonstrated whether they are
coded by the virus genome or are induced cellular polypeptides they are designated 'infected
cell specific polypeptides', i.e. ICSPs. Based on the results of a series of experiments, not all
of which are described here, it is suggested that there are at least five polypeptides which fall
into this category – these are designated ICSP-1 to 5 and their electrophoretic mobilities
relative to the virus particle polypeptides are shown schematically in Fig. 2. Our criterion of
including a polypeptide within this grouping is either that it cannot be discerned in the
uninfected cell or that if there is a polypeptide of similar electrophoretic mobility in the
uninfected cell then its synthesis appears to be shut off at a different rate after infection than
the synthesis of the majority of cellular polypeptide species in the uninfected cell. ICSP-1, 3
and 5 fall into the first category while ICSP-2 and 4 are in the second (and are thus perhaps
of more doubtful significance). A useful means of relating synthesis of polypeptides to those
in the uninfected cells is to compare labelling relative to one of the major cellular poly-
peptides (marked X in Fig. 1). Thus ICSP-2 synthesis is still apparent at times when poly-
peptide X is no longer detectable, although in uninfected cells there is clearly a component
of similar mobility to ICSP-2. Approximate mol. wts for these polypeptides can be cal-
culated with reference to the virus-particle polypeptides which themselves have been
characterized with marker proteins in the SDS acrylamide gel system (Maizel et al. 1968b;
Russell et al. 1971b). These values are given in Table I. (For explanation of terminology of
virus components see Ginsberg et al. (1966) and Russell et al. (1971b).)

Time course of polypeptide synthesis in infected cells

Previous investigations have shown that by infecting cells in suspension at a high multi-
plicity (200 p.f.u./cell), an appreciable degree of synchrony of infection occurs and a
sequential series of events can be detected both by immunological methods and by studying
DNA synthesis (Russell et al. 1967a; Hayashi & Russell, 1968; Mäntyjärvi & Russell,
1969).

A similar approach was used to follow virus polypeptide synthesis by ‘pulse’ labelling
cells at various times after infection for 30 min. with [35S]-methionine. Immediately after the
labelling period the cells were harvested and the cellular extracts prepared for SDS acryla-
mide gel electrophoresis. Extracts of labelled uninfected cells were similarly obtained at the
same time. The results are shown in Fig. 3 and 4. The first experiment (Fig. 3) shows quite
clearly that even at 4 hr after infection one of the ICSP's can be readily distinguished and
indeed ICSP-3 is the only polypeptide easily characterized until 11 to 13 hr post-infection,
when the other ICSP's and the virus-particle polypeptides first become apparent. At the
same time there is a marked decrease in cellular protein synthesis until by about 18 hr most
Fig. 3. Time course of polypeptide synthesis in infected HEK cells. Autoradiograms from uninfected and infected cells when labelled for 30 min, beginning at the post-infection times shown. Gel on the left of each pair shows the pattern obtained from uninfected cells. Also shown are the patterns obtained with labelled purified virus and an extract of infected cells labelled from 12 to 36 hr after infection (IC).
The polypeptides of adenovirus-infected cells

Fig. 4. Time course of polypeptide synthesis in infected HEK cells, soon after infection. Autoradiograms from uninfected (C) and infected cells when labelled for 30 min. beginning at the post-infection times shown (hr). The uninfected cells were obtained from a "mock" infected cell culture labelled for 30 min. at 15 hr post infection. The pattern from purified virus is also shown (V).

of the cellular polypeptide synthesis has been completely abolished. It is also apparent from this experiment that some of the ICSP's are not always synthesized simultaneously with the capsid polypeptides. Thus, ICSP's 2 and 3 and to a lesser extent ICSP-1 are relatively less prominent later in infection. The same is also true of the core polypeptides, particularly core polypeptide 2, which is rich in arginine (Russell et al. 1971b) – this polypeptide is first produced about 13 hr after infection and its synthesis is then relatively diminished at the later times. In this particular figure it is difficult to distinguish ICSP-4 from core polypeptide 1 – in other autoradiograms the distinction is much clearer (e.g. Fig. 5). A further time course experiment was carried out to decide the earliest time after infection at which ICSP 3 could be detected. Fig. 4 shows that as early as 2½ hr after infection the polypeptide could be discerned. This experiment also clearly demonstrates that most of the ICSP's and the core polypeptides are not synthesized later in infection.

The time of appearance of the capsid polypeptides is in reasonable agreement with the
earlier immunological experiments, although these suggested that the penton base antigen was made somewhat later than either the hexon or fibre antigens. In these initial experiments (Russell et al. 1967a) a so-called 'early' antigen, designated 'P' antigen, could be detected at about 6 hr post-infection by using a rabbit antiserum prepared against extracts of rabbit kidney cells infected with adenovirus type 5 in the presence of cytosine arabinoside.

From the results of the time-course experiments it appears fairly likely that the P antigen can be correlated with the ICSP-3 polypeptide. However, a more definitive relationship has been established in the investigations described below. These experiments have mainly
The polypeptides of adenovirus-infected cells

Fibre
Penton base
Hexon

Fig. 6. Autoradiograms of labelled polypeptides after fluorocarbon extraction and after separation of 'nuclear' and 'cytoplasmic' fractions by the detergent method. HEK cells were labelled for 30 min. at 16 hr post infection and divided into two aliquots, one of which was extracted with fluorocarbon and the other treated with detergent. 1, Aqueous phase after fluorocarbon extraction; 2, interphase after fluorocarbon extraction; 3, 'nuclear' fraction; 4, 'cytoplasmic' fraction.

followed previous experimental procedures the results of which have been described in terms of the antigens produced and are now also described in terms of the polypeptides synthesized.

The effect of cytosine arabinoside on polypeptide synthesis in infected cells

On infecting cells with adenovirus in the presence of cytosine arabinoside, virus and cellular DNA synthesis is inhibited but not the synthesis of P antigen (Russell et al. 1967a). A similar experimental protocol was devised to investigate polypeptide synthesis in both HEK and RK 13 infected cells. (The latter cells were chosen since these were used to prepare the original P antigen.) Fig. 5 shows that, in the presence of the inhibitor, although the
cellular polypeptide synthesis is not inhibited, the synthesis of ICSP-3 and either ICSP-4 or core protein-I polypeptide can be detected. These results therefore support the proposition that the P antigen contains ICSP-3 as a major component.

The effect of deprivation of arginine on polypeptide synthesis in infected cells

When adenovirus infects cells growing in arginine-deprived medium, infectious virus is not produced (Rouse & Schlesinger, 1967) and immunological experiments have shown that although a reduced yield of capsid antigens is obtained, the synthesis of P antigen as determined by complement fixation is apparently uninhibited (Russell & Becker, 1968). Fig. 5 shows the distribution of labelled polypeptides from extracts of cells pulse-labelled at 16 hr post infection for 30 min. in the absence of arginine in the tissue-culture medium. It can be seen that under these conditions ICSP-3 is synthesized at a relatively rapid rate and that the capsomere and core-I polypeptides are synthesized at a much slower rate in agreement once again with the immunological data. Of further interest, moreover, is the apparent inhibition of synthesis of the core-2 polypeptide, and that cellular polypeptide synthesis is not inhibited to the same extent as in the arginine-containing medium, e.g. synthesis of polypeptide X is still apparent.

‘Nuclear’ and ‘cytoplasmic’ fractionation of infected cells

In earlier investigations, in an attempt to concentrate and possibly partition virus components, nuclear and cytoplasmic fractions of infected cells were prepared using a detergent method. The results showed that P antigen was preferentially retained in the ‘nuclear’ fraction with a concomitant leakage of the capsomere antigens into the ‘cytoplasmic’ fraction (Mäntyjärvi & Russell, 1969). Fig. 6 indicates that after fractionating cells which were pulse-labelled at 15 hr post infection the ‘nuclear’ fraction preferentially retained polypeptide ICSP-3 and both the core polypeptides in parallel once again with the previous immunological results.

Fluorocarbon extraction

Extraction with fluorocarbon is a standard method of partially purifying adenovirus antigens and an analysis of the fate of the labelled polypeptides after extraction was undertaken. The gelatinous interphase obtained after extraction with fluorocarbon is not amenable to standard antigenic investigation but can be analysed by this electrophoretic technique.

Fig. 6 shows the distribution of the labelled polypeptides between the aqueous phase and the interphase of the two phase system and the result suggests that there might be preferential extraction of some of the polypeptides, e.g. of ICSP-I, into the fluorocarbon interphase.

Immuno-precipitation

From the results shown in Fig. 7 it is evident that although there is some ‘background’ of labelled polypeptides using hexon and control rabbit antisera, the major difference with the P antiserum is the appearance of ICSP-3 in the precipitate. Another notable feature of this experiment is the ‘prozone’ effect at the higher concentration of hexon antiserum. The background of hexon and fibre polypeptides seen is probably due to the non-specific association of these components with the precipitate since no attempt was made to wash the delicate antigen-antibody complexes.
DISCUSSION

These investigations have shown that the technique of labelling cells with high specific activity [35S]-methionine, when combined with SDS acrylamide gel electrophoresis followed by autoradiography, provides an extremely useful tool for analysing the synthesis of polypeptides. In particular, the resolution afforded by the method permits identification of some virus-induced polypeptides even in the presence of normal cellular polypeptide synthesis. The procedure, of course, will only detect methionine-containing polypeptides, and although the major virus polypeptides contain 1.1 to 1.3% methionine (Russell, 1971) it is possible that some virus gene products do not contain this amino acid.
The experiments have clearly demonstrated that as early as 2½ hr after infection a polypeptide of molecular weight $6.4 \times 10^4$, designated ICSP-3, is synthesized which is not incorporated into the virus particle. This polypeptide shows the characteristic property of an ‘early’ antigen or enzyme, i.e. its synthesis is not impeded in the presence of an inhibitor of DNA synthesis and a series of other tests show that this polypeptide is the major component detected by the P antiserum. The fact that P antiserum also reacts with degraded virus and also with core components (Russell & Knight, 1967; Russell et al. 1971b) in complement fixation tests could perhaps be explained by the finding shown in Fig. 4 that possibly the synthesis of core protein-I is not inhibited by cytosine arabinoside. (The P antiserum was prepared by using extracts of rabbit cells infected in the presence of this inhibitor (Russell et al. 1967a)). If this were the case then core polypeptide-I could also be classified as an ‘early’ product. Fig. 4 also demonstrates that the abortive infection of rabbit cells with adenovirus even in the absence of inhibitor yields a strikingly different pattern of polypeptide synthesis from the normal lytic infection. There is a relatively poor synthesis of the virus-particle polypeptides compared to the synthesis of ICSP’s (particularly ICSP-1 and 3).

Fig. 4 further shows that in the absence of arginine in the tissue culture medium the arginine-rich core-2 polypeptide is not made – a conclusion which had been reached using more indirect immunological and cytochemical techniques (Russell & Becker, 1968; Russell Brodaty & Armstrong, 1971a; Russell et al. 1971b). It is interesting to note that in the current experiments there is no clear indication that the P antiserum reacts with antigens containing core-2 polypeptides and it may be that any relationship between this antiserum and core-2 protein follows from the association of this core protein with core-1 protein which may be detected by the P antiserum (cf. Russell & Becker, 1968; Russell et al. 1971a).

Another major result of this investigation is that it indicates means of detecting those polypeptides which are not virus-particle components but appear to be specific for the infected cells. It will be clearly necessary to attempt to separate and further characterize these components and preferential fractionation procedures such as fluorocarbon extraction and the preparation of cellular fractions as described may provide useful first steps in the separation procedures. Preliminary pulse-chase experiments have so far not demonstrated any precursor product relationships between the polypeptides, although more detailed experiments will be required to clarify this point (cf. Horwitz, Scharff & Maizel, 1969). If all of the ICSP’s designated here are indeed virus gene coded then about 70% of the total potential coding capacity of the virus DNA can be accounted for (cf. Russell, 1971). In this connexion it should also be pointed out that SDS gel acrylamide electropherograms will not distinguish very clearly polypeptides of similar molecular weights and indeed in some experiments there have been suggestions that some of the bands designated in the ICSP category may indeed contain multiple components of similar molecular weights. If this is shown to be the case then even more of the coding capacity of the virus genome can be accommodated.

It is interesting to note that if all the ICSP’s can be classified as separate virus gene products then about 10 to 15% of the virus polypeptides can be classified as ‘early’, in reasonable agreement with the studies of Green and his colleagues who have shown that about one-fifth of the virus genome is transcribed early, i.e. before virus DNA synthesis (Green, 1970).

The function of all these ICSP components in the process of infection is unknown but the methods described here offer an opportunity of characterizing them further.
The polypeptides of adenovirus-infected cells

We have pleasure in acknowledging the skilled technical assistance of Mrs Carol Newman and Messrs David Stevens and John Wills. Autoradiography and photography were carried out with the willing assistance of members of the Photography Department of the Institute. The advice of Dr H. G. Pereira is also appreciated.

REFERENCES


(Received 18 November 1971)