Characterization of Adenovirus Protein IX

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SUMMARY

Protein IX from adenovirus type 2 was purified by two methods, one from groups of nine hexons obtained by disrupting purified virus by heating in the presence of deoxycholate, and the other by a previously published method. The purified protein was used to obtain a monospecific antiserum. Protein IX was found to possess both sub-group- and type-specific antigenic determinants which were apparently accessible within the groups of nine hexons. Approximately 15 molecules of IX were found per group of nine hexons and from considerations of symmetry it seemed possible that IX was located at the ‘corner to edge’ contacts between hexons in the icosahedron.

The protein in infected cells was found to possess approximately neutral charge as determined by immunoelectrophoresis. This was consistent with the amino acid composition, which showed it to be rich in serine, alanine and leucine with approximately half of its glutamic and aspartic acid residues amidified, and the isoelectric point of 6.0, as determined by two dimensional gel analysis. No free N-terminal amino acid was detectable. It is suggested that a unique tryptophan residue is located at around position 70 from the blocked N-terminus, on the basis of chemical cleavage by BNPS-skatole. Based on one tryptophan residue a total of 107 amino acids and a mol. wt. of 11 200 was deduced. Analysis of 35S-methionine-labelled infected cell extracts in a two-dimensional gel system showed that the synthesis of polypeptide IX could be detected early in infection, i.e. in the presence of an inhibitor of DNA synthesis.

INTRODUCTION

The icosahedral adenovirus virion consists of a linear double stranded DNA in association with at least ten different structural protein components. The topology of these protein components is not very well understood (Philipson & Lindberg 1974; Nermut, 1975) although it is fairly clear that the main component of the capsid, the hexon, is present in association with the other capsid proteins in two different configurations. Thus the hexons contiguous to the apices of the icosahedron (the peripentonal hexons) have a different environment from those at the faces and edges of the icosahedron (Pereira & Wrigley, 1974). These latter hexons appear to be associated with each other in groups of nine hexons with threefold symmetry (Crowther & Franklin, 1972) as revealed by disruption of the virus (Smith et al. 1965; Russell et al. 1971). It has been assumed that these groups of nine hexons were held together by association with at least one of the other structural proteins

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of the virion. In the work described here it is suggested that polypeptide IX fulfils this role. Recent work elsewhere (Pettersson & Mathews, 1977; Persson et al. 1978) has indicated that this polypeptide is coded by a region in one DNA strand towards the left hand of the conventional map and moreover appears to be transcribed and translated early in infection, although its peak of synthesis appears after virus DNA synthesis has begun. Our work on the biosynthesis of protein IX supports these conclusions.

In the light of these findings it thus seems possible that in addition to a purely structural role, protein IX may exert some regulatory function early in infection and consequently a more detailed antigenic and biochemical analysis of the protein was undertaken.

METHODS

Cells and viruses. Human adenovirus types 2 and 5 were propagated in KB cells maintained in spinner culture in Eagle’s basal medium supplemented with 5% horse serum.

For in vivo labelling experiments HeLa cell monolayers were grown in Eagle’s minimum essential medium supplemented with 10% calf serum.

Labelling conditions. Adenovirus was labelled by adding 14C-valine (250 to 300 mCi/mmol; Amersham, England) from 18 to 30 h p.i. at 0.5 μCi/ml in a culture medium containing 10% of the concentration of valine in normal medium. Virus was extracted and purified by conventional techniques (Green & Pina, 1963). Pulse-labelling was performed in HeLa cell monolayers, mock-infected or infected with adenovirus at a m.o.i. of 100 to 200. L-35S-methionine (50 to 100 μCi/ml, 700 to 800 Ci/mmol, Amersham, England), was added for 30 min at different times p.i. in a methionine-deprived medium and the cells were harvested just after the pulse (Russell & Skehel, 1972).

Purification of adenovirus type 2 protein IX. Two different methods were used to purify protein IX from adenovirus type 2.

Method 1. An adenovirus suspension (3 to 5 mg protein per ml), purified by two consecutive CsCl equilibrium centrifugations was dialysed against 5 mM-tris-HCl buffer, pH 7.8, and dissociated by heating at 56°C for 90 s with 0.5% (w/v) sodium deoxycholate. The dissociation was accompanied by the sudden disappearance of the opalescence of the virus suspension (Russell et al. 1970. The cleared virus suspension (1 to 1.5 ml) was then layered on top of an 11 ml, 10 to 40% (v/v) glycerol gradient (in 5 mM-tris-HCl buffer, pH 7.8, containing 1.5 mM-Na EDTA) with a 1 ml cushion of 80% glycerol at the bottom and centrifuged at 35000 rev/min and 4°C for 80 min (MSE 6 × 14 ml). Fractions of 0.6 ml were collected dropwise from the bottom, monitored by u.v. adsorption at 260 and 280 nm and analysed by SDS-polyacrylamide gel electrophoresis as described below.

The fractions which contained groups of nine hexons (as judged by the change in absorbance at 280 nm compared to nucleoprotein cores, and by electron microscopy) were pooled and dialysed against 8 M-urea buffered with 10 mM-tris-HCl, pH 8.6, 1.5 mM Na EDTA. After extensive dialysis, the sample was heated at 100°C for 2 min to disrupt the groups of nine hexons and to dissociate the constituent polypeptides.

The sample was then dialysed against 0.1 M-tris-phosphate buffer pH 8.6, containing 4 M-urea and 0.1% (v/v) 2-mercaptoethanol, and loaded (4 to 5 mg of protein) on a 25 ml column of QAE-A25 Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden). The excluded peak, containing solely the protein IX, which does not adsorb to the anion exchange material under these conditions (Everitt & Philipson, 1974), was dialysed against distilled water and concentrated by freeze-drying. The other component, hexon polypeptide II, was retained on the ion-exchange material. The freeze-dried sample of protein IX was dissolved in 50 mM-tris-HCl, pH 7.8, containing 10% glycerol, at a concentration of 0.1 mg of protein per ml.

Method 2. This method was the procedure described by Everitt & Philipson (1974).
It was used to obtain relatively greater amounts of protein IX for biochemical purposes and to compare with method I.  

_**Antisera.**_ Rabbit monospecific antiserum against protein IX was prepared as follows: purified protein IX obtained by method I, at a concentration of 0.1 mg/ml, was mixed with an equal vol. of Freund’s complete adjuvant and injected subcutaneously into a rabbit in 10×50 μl aliquots at a time at 7 day intervals. After 2 months, the animal was bled. Monospecific serum against the hexon antigen of Ad 2 was prepared in rabbits by injection of purified hexons (Boulanger & Puvion, 1973). Polyspecific antiserum towards Ad 2 soluble antigens was prepared by injecting rabbits with an ammonium sulphate precipitate of Ad 2-infected KB cell Freon extracts (Boulanger & Puvion, 1973).

**Polyacrylamide gel analysis**

_Two dimensional analysis_ (isoelectric focusing/SDS gel electrophoresis) was performed according to the method of O’Farrell (1975), with certain modifications. HeLa cell extracts were prepared by sonicating 5×10^6 labelled cells in 0.5 ml lysis buffer [9.5 M-urea, 2%, w/v, NP40, 5%, ampholines (consisting of 3.4%, pH 5 to 7, and 1.6%, pH 3.5 to 10), 5% 2-mercaptoethanol] supplemented with 0.5% SDS. The sonicated extracts were centrifuged for 3 min in an Eppendorf microcentrifuge. Samples (10 to 25 μl) of the supernatant, containing at least 250000 ct/min, were applied to the first-dimension isoelectric focusing gel. This gel was prepared as described by O’Farrell (1975) except that the ampholine concentration was increased to 5%, consisting of a 2:1 ratio of pH 5 to 7 to pH 3.5 to 10 ampholines (LKB, Sweden). Gels were focused at 400 V for 19 h. The second dimension SDS gel was prepared as described below.

Isoelectric points of proteins were estimated by reference to the pH gradient determined by analysis of a parallel isoelectric focusing gel. This gel was divided into 1 cm pieces and incubated in 2 ml de-ionized, de-gassed distilled water for 1 h at 22 °C. The pH of each sample was determined in a standard pH meter.

_Analytical SDS-polyacrylamide gel electrophoresis._ Samples were dissolved in an equal vol. of sample buffer and heated for 2 min at 100 °C. Sample buffer consisted of 0.0625 M-tris-HCl buffer, pH 6.8, containing 6 M-urea, 4% sodium dodecyl sulphate (SDS) and 10% 2-mercaptoethanol. Polypeptides were analysed in an SDS-containing 15.5% polyacrylamide gel (acylamide:bisacrylamide ratio of 50:0.235) overlaid by a 5% spacer gel (acylamide:bisacrylamide ratio of 50:1:33) in a discontinuous buffer system (Russell & Blair, 1977). In certain cases gels were impregnated with PPO (1,5-diphenyloxazole) and exposed to pre-flashed Fuji RX film (Bonner & Laskey, 1974).

**Immunological tests.**

_Double immunodiffusion_ was performed in 0.9% agarose (Industrie Biologique Française, France) containing 0.14 M-NaCl and buffered with 0.02 M-sodium barbital, pH 8.6.  

_Immunoelectrophoresis_ was performed at 3 V/cm for 2 h at room temperature in 0.9% agarose buffered with 0.02 M-sodium barbital, pH 8.6.

_Two-dimensional immunoelectrophoresis_ was carried out according to a modification of the basic technique of Laurell (1965), which has been described in detail elsewhere (Martin et al. 1975).

_Immune precipitation._ Labelled HeLa cells (5×10^6) were scraped and washed in tris-buffered saline (35 mM-tris-HCl, pH 7.5, 150 mM-NaCl), resuspended in 0.5 ml extraction buffer [20 mM-tris-HCl (pH 7.5), 10 mM-NaCl, 0.5% Triton X-100, 2 mM-PMSF] and sonicated with three bursts of 5 s each in a probe sonicator (MSE Ltd.) at low power. The NaCl concentration was then raised to 150 mM and the extracts centrifuged for 3 min in a microfuge. Samples (100 μl) of the supernatant were mixed with 100 μl NET.N buffer.
(50 mM-tris-HCl, pH 7.5, 150 mM-NaCl, 5 mM-EDTA, 0.05% NP40, 0.02% sodium azide) supplemented with 0.2% bovine serum albumin and 20 μl normal rabbit serum. Incubation was for 18 h at 4 °C.

Immune complexes were collected by adsorption to inactivated Staphylococcus aureus cells (Cowan I strain; Kessler, 1975). A sample (50 μl) of a 10% (w/v) washed cell suspension (in NET.N buffer) was added to the precipitation reaction and incubated for 30 min at 22 °C. Cells were pelleted by centrifugation in the microfuge. Anti-Ad 2 IX serum (20 μl) was added to the supernatant and incubated for a further 24 h at 4 °C. Immune complexes were again collected by adsorption to S. aureus cells and washed three times in NET.N buffer. Labelled proteins were eluted from the S. aureus cells by incubation with 20 μl of 1% SDS, 0.02 M-DTT for 1 h at 22 °C (Crawford & O'Farrell, 1979). This sample was analysed in the two dimensional system. The cells were removed by centrifugation and solid urea was added to the supernatant to 9.5 M followed by 10 μl of stock NP40, 5 μl 1 M 2-mercaptoethanol and 20 μl lysis buffer.

The preparation of immunoprecipitates for one-dimensional SDS gel analysis was essentially similar to that described above, except that cells were disrupted by three cycles of freezing and thawing and the precipitation step with normal serum was omitted. The final S. aureus cell pellet was resuspended in 50 μl sample buffer, heated for 2 min at 100 °C and applied directly to the SDS gel.

**Amino acid analysis.** Samples of protein IX corresponding to 0.2 to 0.25 mg of protein were hydrolysed for 24 h and 72 h at 110 °C with 5.6 N-HCl, in the presence of tryptamine (1 mg/ml) to preserve tryptophan (Liu & Chang, 1971) and analysed in a Beckman Multichrom amino acid autoanalyser. Amide content was determined on total enzymic hydrolysates, as previously described (Boulanger et al. 1978a).

**N-terminal acid determination** was performed according to the method of Casola et al. (1974), using 14C-DNS-Cl (dansyl-chloride; 104 mCi/mmol, 0.5 mCi/ml; C.E.A., Saclay, France). Autoradiographs of two-dimensional thin layer plates were prepared by exposure to Kodak ‘Kodirex’ film.

**Protein concentration** was assayed by the method of Lowry et al. (1951), using bovine serum albumin as standard.

**Iodination of protein IX.** Purified native IX was labelled with 125I by the chloramine-T method (Hunter & Greenwood, 1962), giving a sp. act. of 5 to 10 × 10⁶ ct/min/μg of protein.

**Chemical cleavage of protein IX at tryptophan residue(s).** A sample of protein IX, labelled with 125I, was treated with BNPS-Skatole (Pierce Chemicals, Rockford, Ill., U.S.A.) according to the method of Eylar et al. (1974) with exogenous tyrosine in 100-fold excess over its tyrosine content. The cleaved protein sample was freeze-dried and analysed by SDS-polyacrylamide gel electrophoresis and autoradiography.

**Nomenclature.** The nomenclature proposed by Ginsberg et al. (1966) for the major capsid components (hexon, penton base and fibre) is used. The remainder of the structural polypeptides are referred to according to the terminology proposed by Maizel et al. (1968) and Anderson et al. (1973) as discussed in relation to adenovirus type 5 by Russell & Blair (1977).

**RESULTS**

**Purification of adenovirus 2 protein IX**

As shown in Fig. 1, the groups of nine hexons, isolated in a glycerol gradient after disruption of adenovirus capsids by heating in the presence of deoxycholate, were composed of two predominant species of polypeptides: II, the hexon polypeptide, and polypeptide IX. After disintegration of the groups of nine hexons by heating in the presence of 8 M-urea, polypeptide IX was readily separated from II; on QAE-A25 Sephadex, protein IX
Fig. 1. Isolation of groups of nine hexons from deoxycholate-disrupted adenovirus on a 10 to 40 % (v/v) glycerol gradient. (a) Absorbance profile at 260 (○—○) and 280 nm (●——●). Fractions 11 to 16 contained the groups of nine hexons. (b) Electrophoretic analysis on an SDS-polyacrylamide gel of the glycerol gradients fractions of (a). Proteins were stained with Coomassie brilliant blue. Ad2 = marker adenovirus type 2.
Fig. 2. Analytical SDS-polyacrylamide gel electrophoresis of protein IX at different stages of purification. (a) Adenovirus type 2; (b) groups of nine hexons (prepared by method 1); (c) purified protein IX obtained by method 1 (protein of identical purity was obtained by method 2); (d) high salt–alkaline–urea extract of adenovirus (method 2). Proteins were stained with Coomassie brilliant blue.

did not absorb to the ion-exchange material (Fig. 2). The final yield was relatively high: from 20 mg of adenovirus particles, 0.25 to 0.30 mg of homogenous protein IX was obtained. This purified protein IX was used to prepare the rabbit serum.

The second method (Everitt & Philipson, 1974) consisted of sequential extraction of proteins from the virion, using high salt–acid–urea treatment, followed by high salt–alkaline–urea extraction. Fig. 2 shows the analytical SDS-polyacrylamide gel electrophoresis of the high salt–alkaline–urea extract, which was the source of protein IX. At this stage the protein was contaminated by the capsid proteins hexon, penton base, IIIa and fibre. Protein IX was separated from the major capsid proteins by ion-exchange chromatography on QAE-A25 Sephadex. From 90 mg of purified virions, 13 to 15 mg of high salt–alkaline–urea extract was obtained, with a final yield of 1.0 to 1.2 mg of pure protein IX.

**Immunoelectrophoretic analysis of protein IX**

When a $^{35}$S-methionine-labelled adenovirus type 2-infected cell extract was immunoelectrophoresed against an anti-Ad 2 soluble antigen serum, several precipitation lines could be distinguished (Fig. 3b); the most anodic line corresponded to hexon antigen, penton and
Fig. 3. Immunoelectrophoretic analysis of adenovirus type 2 protein IX. An extract of adenovirus-infected HeLa cells, labelled from 18 to 24 h p.i. with $^{35}$S-methionine, was loaded in the central well and electrophoresed as indicated in Methods. The dried plate was exposed to X-ray film. (a) Monospecific serum anti-IX; (b) polyspecific serum anti-Ad2 soluble antigens; H, hexon antigen; P, penton; F, fibre.

Fig. 4. Crossed immunoelectrophoresis of adenovirus type 2 protein IX. A $^{35}$S-methionine-labelled adenovirus type 2-infected HeLa cell extract was electrophoresed in a first dimension from left to right, then at right angles in a second dimension in an antiserum-containing agarose gel. H, hexon; PB, penton base; P, complete penton; F, fibre. (a) Polyspecific serum anti-Ad2 soluble antigens; (b) monospecific serum anti-adenovirus type 2 IX. The dried plate was exposed to X-ray film. Loads: 10 $\mu$l of cell extract; antisera: 100 $\mu$l in each plate.

fibre antigens being more cathodic. The monospecific serum anti-protein IX revealed a single precipitation line with an electrophoretic position more cathodic than that of fibre antigen (Fig. 3a). This cathodic antigen was also revealed by the polyspecific antiserum.

Two-dimensional immunoelectrophoresis of protein IX

The $^{35}$S-methionine-labelled Ad 2-infected cell extract was cross immunoelectrophoresed against the polyspecific serum anti-Ad 2 soluble antigens (Fig. 4a) and the monospecific anti-protein IX serum (Fig. 4b). No immune precipitate peak was revealed by the
anti-IX serum, but a precipitation line was visible on the autoradiogram of the two-dimensional plate in the vicinity of the loading well. Fig. 4(b) also revealed that no antibody against hexon, penton base or fibre was present in the monospecific anti-IX serum: no precipitate formed with either of these antigens, but spots with a diagonal migration were seen on the two-dimensional plate autoradiogram. Such spots would be expected if antigens moved freely in an electric field.

**Antigenicity of protein IX and relationship between protein IX of adenovirus serotypes 2 and 5**

Adenovirus type 2- and type 5-infected cell extracts were tested by double immunodiffusion against the anti-IX serum. Fig. 5(a) shows that anti-IX serum revealed one precipitation line against both types 2 and 5 unlabelled extracts, with a spur suggesting group- and type-specific determinants. Fig. 5(b) shows the double immunodiffusion pattern obtained with extracts of type 2- and type 5-infected cells labelled with $^{35}$S-methionine from 18 to 24 h p.i. Anti-IX serum revealed two labelled precipitation lines against the type 2 extract: the thinnest one, which was also the nearest to the central well containing the immune serum, formed a continuous line with a corresponding antigen present in the type 5 extract. The presence of antibody against hexon antigen seemed unlikely since this class of antibody was not detected in the two-dimensional pattern (Fig. 4b). Furthermore, the anti-IX serum had been pre-incubated for 4 h at 37 °C with purified hexon antigen.

The apparently unique precipitation line of the type 2 extract visible in agarose (Fig. 5a) most likely represented the superimposition of the two lines detected in the labelled extract (Fig. 5b). This pattern of antigen-antibody precipitation lines suggested that protein IX existed in different forms in the two cell extracts and that these forms were antigenically distinct in adenoviruses belonging to these serotypes. Alternatively, the thickest precipitation line, revealed in type 2 extract, might represent aggregates or oligomers of protein IX, which failed to occur in the type 5 extract. Such oligomeric forms of adenovirus 2 protein IX have been previously suggested (Everitt & Philipson, 1974).

Analysis of immune complexes formed between type 2 and type 5 infected labelled cell extracts and anti-type 2 protein IX antibody, revealed that polypeptide IX of both types 2 and 5 was precipitated by the antibody (Fig. 6).
Fig. 6. Immunoprecipitation of protein IX of adenovirus types 2 and 5 with the anti-type 2 IX rabbit immune serum. Immunoprecipitation with anti IX serum was performed as described in Methods. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis. (a) Adenovirus type 2-infected HeLa cell total extract pulse-labelled with $^{35}$S-methionine at 18 h p.i.; (b) an immunoprecipitate of the same extract obtained with anti-type 2 IX serum; (c) an immunoprecipitate of type 5-infected cell late extract (18 h p.i.) obtained with anti-type 2 IX serum; (d) a mock-infected HeLa cell extract reacted with anti-type 2 IX serum. Equivalent ct/min were present in all the incubation mixtures. Traces of hexon, 100 K, and other capsid proteins were detected which presumably adsorbed non-specifically to *S. aureus* cells. These proteins are major proteins in the infected cell compared to polypeptide IX. Adenovirus type 5 hexon could be distinguished by its lower mol. wt. (115000) than that of the type 2 hexon polypeptide (120000).

**Antigenicity of protein IX within groups of nine hexons**

The availability of the antigenic determinants of the virion protein IX in the nonameric edifice was examined. Two-dimensional analysis of groups of nine hexons was carried out by cross immunoelectrophoresis with antiserum against hexon antigen and against antiserum towards protein IX. This revealed that IX antigenic determinants were accessible in groups of nine hexons, the immune precipitate peak of IX-anti-IX antibody being visible in the same position as the hexon–anti-hexon precipitates (Fig. 7). If this result had been due to small amounts of anti-hexon antibody contaminating the anti-IX serum a high, faintly stained precipitation peak with no top of the peak visible on the plate would have been
Fig. 7. Two-dimensional immunoelectrophoretic analysis of protein IX within groups of nine hexons. Groups of nine hexons obtained after deoxycholate-disruption of adenovirus particles (prepared as in fraction 13 of Fig. 1a) were cross immunoelectrophoresed against either anti-hexon serum (a), or anti-protein IX serum (b). Loads: 5 μl of antigen and 75 μl of antiserum (a); 20 μl of antigen and 200 μl of antiserum (b).

expected. In this case, the equivalence point between major hexon antigen present in groups of nine and the minor population of hexon antibody would have occurred after a relatively long migration in the second dimension, sufficient to allow antigens to encounter a significant number of antibody molecules (Laurell, 1965). The two-dimensional pattern shown in Fig. 7(b) indicates a relatively high level of antibody in the antiserum and a low level of antigen in the groups of nine hexons.

Tryptic digestion (0.1 mg of enzyme per 0.5 mg of protein) of groups of nine hexons retained the antigenicity of protein IX, as demonstrated by the persistence of the precipitation line in double diffusion tests (data not shown). However, nicks were produced in the polypeptide chain of IX, since SDS-polyacrylamide gel electrophoresis of trypsin-digested groups of nine hexons showed that protein IX as such had disappeared and had been replaced by cleavage fragments (data not shown).

Composition of protein IX per virion and per hexon nonamer

Adenovirus particles were labelled with 14C-valine, an amino acid apparently almost equally represented in all virion proteins (Philipson & Lindberg 1974). Freshly purified virions were denatured by heating in SDS and electrophoresed in an SDS-polyacrylamide gel. Autoradiographs of such gel tracks were scanned in an automatic cellulose-strip scanner (Quick Quant II autoscanner, Helena Lab Corp. Beaumont, Tx, U.S.A.). The composition of polypeptide IX was found to be 2.4 to 2.5% of the total protein of the virion. Thus total protein, based on the DNA content (12 to 13%) and its mol. wt. (23 × 10^6), has been estimated to about 150 × 10^6 (Green, 1970). Assuming a mol. wt. of 12500 for for protein IX (Anderson et al. 1973), a value of 288 molecules of IX per virion could be deduced.

Similarly, scanning of protein components of groups of nine hexons electrophoresed in an SDS-polyacrylamide gel (as in Fig. 1b) yielded a value of 18.8 to 19.3 for the ratio of polypeptide II to polypeptide IX, i.e. a mean value of 13.6 molecules of IX per hexon.
Adenovirus protein IX

Fig. 8. Immunoprecipitation of adenovirus type 2 protein IX in productive infection. Infected HeLa cells were pulse-labelled with 35S-methionine for 30 min between 0 (mock-infected cells) and 24 h p.i. The cells were harvested after the pulse and disrupted by freezing and thawing. Immunoprecipitation was performed as described in Methods and samples were analysed by one-dimensional SDS-polyacrylamide gel electrophoresis. Labelling times p.i. were 0, 4, 6, 8, 10, 12, 18, 24 h; infected cell total extract (i) was obtained at 18 h p.i. Due to the overloading of protein and nucleic acid material, the migration of high mol. wt. polypeptides such as II and 100K is slightly retarded in slot i.

nonamer, assuming a mol. wt. of 360000 for the hexon capsomer. There are 20 groups of nine hexons in the adenovirus shell and the nearest integral number of polypeptide IX molecules would be 14 per hexon nonamer, and therefore 280 per virion.

**Time-course of synthesis of Ad 2 protein IX in HeLa cells**

HeLa cell monolayers (6 × 10⁶ cells per bottle), mock-infected and infected with adenovirus type 2 were pulse-labelled for 30 min with 35S-methionine at 4, 6, 8, 10, 12, 18 and 24 h p.i. After the pulse the cells were harvested, disrupted by three cycles of freezing and thawing in hypotonic buffer (10 mM-tris-HCl, pH 7.8, containing 1.5 mM-2-mercaptoethanol, 2 mM-Na EDTA and 1 mM-PMSF) and centrifuged at 1000 g for 5 min.

Samples of each supernatant were incubated with different antisera and immunoprecipitates were analysed on a one-dimensional SDS-polyacrylamide gel. Fig. 8 shows the results obtained with the anti-IX serum. Protein IX could be discerned only at 9 to 10 h p.i.,
Fig. 9. Two dimensional gel analysis of polypeptide IX. Extracts of mock-infected HeLa cells (a) and HeLa cells infected with adenovirus type 5 in the presence of 25 μg/ml cytosine arabinoside (b) were analysed on two dimensional gels (as described in Methods). Cells were pulse-labelled with 35S-methionine (100 μCi/ml) at 18 h p.i. In a separate experiment, an immunoprecipitate of polypeptide IX prepared with anti-type 2 IX serum and an extract of 'late' type 5-infected cells (i.e. prepared at 18 h p.i. in the absence of cytosine arabinoside) was also analysed in the two-dimensional system (c). V=35S-methionine-labelled adenovirus type 5, electrophoresed in the second dimension gel to serve as a reference marker. Gels were impregnated with PPO, dried and exposed to pre-flashed X-ray film for 10 days.
Adenovirus protein IX

with a maximum of synthesis at 20 to 24 h p.i. Traces of hexon polypeptide and of protein 100 K were visible, possibly as a result of non-specific trapping with the immune complexes on *S. aureus* cells. No newly synthesized protein IX could therefore be detected in the absence of hexon polypeptide synthesis in these one-dimensional gels, using labelled extracts of adenovirus type 2 (Fig. 8) or type 5 infected cells (results not shown).

Two-dimensional gel analysis

In order to obtain more definitive evidence on the time of synthesis of polypeptide IX, two-dimensional gel analysis (isoelectric focusing/SDS gel electrophoresis) was performed. A ^35S^-methionine pulse-labelled extract of HeLa cells infected with adenovirus type 5 in the presence of cytosine arabinoside (an inhibitor of DNA replication) was prepared, to obtain an 'early' pattern of virus protein synthesis (Fig. 9b). This analysis revealed the synthesis in infected cells of a protein species which possessed an apparent isoelectric point (pI) of 6.0 and migrated with a mol. wt. of approx. 12 K. This species was not present in mock infected cells (Fig. 9a). There was also no evidence for multiple forms of the species focusing at different pH values, suggesting that no charged groups (e.g. phosphate groups) are added to the protein after translation.

Further characteristics of the 'early' proteins detected by this analytical method will be published elsewhere. However, the presence of a 72 K mol. wt. species was readily observed (Fig. 9b). This species is presumably identical to the virus-coded single stranded DNA-binding protein, a characteristic 'early' virus protein. No evidence of the synthesis of other structural proteins (e.g. hexon) was obtained.

The identity of the pI 6.0/12 K species with polypeptide IX was confirmed in a separate experiment of immunoprecipitation using either 'early' or 'late' adenovirus type 5-infected cell extracts and anti-Ad 2 IX serum. The immunoprecipitate from 'late' infected cells was analysed in the two-dimensional system (Fig. 9c) and revealed a spot of identical mobility in both dimensions to that of the spot observed in infected cells blocked in DNA replication (Fig. 9b). Identical results were obtained from 'early' infected cells except that the intensity of the polypeptide IX spot was greatly diminished (results not shown). This suggests that late in infection, the rate of synthesis of polypeptide IX increases. This analytical approach was repeated using 'early' and 'late' type 2-infected cell extracts and quantitatively identical results were obtained. The only difference appeared to be that the type 2 polypeptide IX possessed a slightly more acidic pI than the type 5 protein (results not shown).

We conclude that IX is synthesized at a relatively low rate 'early' in infection, before virus DNA replication takes place and in the absence of other detectable structural proteins.

Amino acid composition of protein IX

Amino acid analysis of protein IX purified by the second method was carried out. As previously reported (Everitt & Philipson, 1974) protein IX showed a high content of serine and alanine. Serine, alanine and leucine accounted for more than 40% of the total amino acid content of the protein (Table 1). A very small amount of cysteine, histidine and tryptophan were found. Based on one tryptophan residue, a total number of 107 residues could be obtained with a calculated mol. wt. of 11,200, suggesting that the polypeptide indeed contained unique cysteine, histidine and tryptophan residues. The total acid hydrolysis revealed an excess of aspartic and glutamic acid residues over the basic amino acids. However, analysis of total enzymic hydrolysates showed that in fact more than 50% of the side-chain carboxylic groups were amidified (Table 1), there being only four aspartic acid versus five asparagine, and only three glutamic acid versus four glutamine residues. This could account for the approximate neutrality of protein IX, already noted from its migration in two-dimensional gels and in immunoelectrophoretic tests.
Table I. *Amino acid composition of adenovirus 2 protein IX*

<table>
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<tr>
<th>Amino acid*</th>
<th>Mol. per 100 mol. of all amino acids recovered</th>
<th>Residues per 1 Trp</th>
<th>Amide content† (%)</th>
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<td>9</td>
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<td>8·7</td>
<td>9</td>
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<tr>
<td>Ser‡</td>
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<td>15</td>
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</tr>
<tr>
<td>Ile§</td>
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<td>2</td>
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<tr>
<td>Leu§</td>
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<tr>
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<tr>
<td>Phe</td>
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<td>3</td>
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<tr>
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<tr>
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<tr>
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<td>1</td>
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</tr>
<tr>
<td>Arg</td>
<td>5·5</td>
<td>6</td>
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</table>

* Average of two determinations on 24 h and 72 h acid hydrolysates.
† Determined from the difference between aspartic and glutamic contents in acid and total enzymic hydrolysates. Figures in parentheses indicate the closest integral number of asparagine and glutamine per 1 residue of tryptophan. No cysteic acid was detected and all the half-cystine was recovered in the peak of cystine.
‡ Extrapolated to zero time hydrolysis.
§ Based on 72 h hydrolysis value.

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**Fig. 10. SDS polyacrylamide gel electrophoretic analysis of the cleavage products of adenovirus type 2 protein IX with BNPS-Skatole.**

- (a, f, g, k) Control 14C-valine-labelled adenovirus 2;
- (b, c, h) 125I-labelled protein IX;
- (d, e) 125I-labelled protein IX cleaved with BNPS-Skatole;
- (i, j) BNPS-Skatole-cleavage products digested with amino-peptidase M;
- (a to f) and (g to k) were two separate experiments.
Adenovirus protein IX

N-terminal amino acid

No free N-terminal amino group was found available for dansylation with $^{14}$C-DNS-Cl, suggesting a blocked N-terminus in protein IX.

Position of the unique tryptophan residue in the polypeptide chain of IX

Protein IX (0.010 mg of protein) was labelled with $^{185}$I and cleaved with BNPS-Skatole. After freeze-drying, the resulting cleavage products were analysed by electrophoresis in an SDS-containing 20% polyacrylamide gel followed by autoradiography. Inspection of Fig. 10 and also densitometric scanning (not shown) revealed that the polypeptides resulting from the chemical cleavage migrated as two blurred bands of apparent mol. wt. 7500 (7.5 K) and 4500 (4.5 K). The same products were subjected to aminopeptidase M digestion (amino acid arylamidase, Boehringer, Mannheim) in order to determine which one of the two polypeptides carried the blocked N-terminus: the polypeptide 7.5K seemed to be resistant to aminopeptidase digestion, whereas the 4.5K band disappeared (Fig. 10). This suggested that the unique tryptophan residue was located around position 70 in the polypeptide chain of IX, starting from the N-terminus.

DISCUSSION

It has been claimed that IX is an acidic protein (Everitt et al. 1973) and this was apparently confirmed by the amino acid composition and the ratio of acidic to basic amino acids (Everitt & Philipson, 1974). However these authors did not determine the amide status or the tryptophan composition of the protein. Our amino acid composition of IX showed approximately equivalent numbers of basic and acidic amino acids and the results of our total enzymic hydrolysis showed that about 55% of the aspartic and glutamic were amidified (Table 1). The two dimensional analysis of labelled polypeptides allowed us to assign a value of 6.0 for the isoelectric point of polypeptide IX. Given the error involved in both analytical systems, it seems reasonable to assume that IX is a protein of approximately neutral isoelectric point. This assumption would explain the failure of IX to migrate significantly at pH 8.6 in our immuno-electrophoretic analyses (Fig. 3 and 4).

An interesting feature was also the large amount of serine (15%), alanine (16%) and leucine (11.5%) in protein IX (Everitt & Philipson, 1974). Despite the high serine content, no evidence was found for the presence of phosphorylated derivatives of the protein by two-dimensional gel analysis, in agreement with previous results (Russell & Blair, 1977; Blair & Russell, 1978). Unique histidine, cysteine and tryptophan residues were also found. Protein IX was found to have a blocked N-terminus, suggesting that it is a primary translation product (Lewis et al. 1975).

The group of nine hexons constitutes a face and part of the three adjacent edges of the icosahedron. It has been shown to have threefold symmetry (Crowther & Franklin, 1972). Stoichiometric analysis of protein IX in the virion and in the group of nine hexons suggested a number in the region of 14 molecules of protein IX per hexon nonamer. The only way to preserve the threefold symmetry of the group of nine hexons appears to be to place each of these IX proteins at each contact between two hexon capsomers, termed 'corner-to-edge' bonds, the hexon capsomer being a trimeric molecule (Nermut, 1975). There are 15 contacts of that sort within the group of nine hexons, and a tentative model of the location of protein IX is presented in Fig. 11. In this model, protein IX is represented as buried in the external side of the capsid, protruding inwards.

The model is based on the following experimental observations: (1) the accessibility of antigenic determinants of IX to antibody in groups of nine hexons; (2) the sensitivity of the protein IX polypeptide chain to tryptic digestion in groups of nine hexons; (3) the difficulty
Fig. 11. Tentative topological model for protein IX within a group of nine hexons. Two groups of nine hexons are represented contacting at an edge ('edge-to-edge' bonds). Triangles represent hexon capsomers. Filled circles and bars represent proteins IX, located at the 'corner to edge' contacts between the hexons. (a) Upper view; (b) transverse section of capsomers; o, outer side of the capsid; i, inner side of the capsid. Modified, after Nermut (1975).

of enzymic iodination of IX in intact virions (Everitt et al. 1975), in contrast to its efficient iodination by chemical procedures (P. Boulanger & W. C. Russell, unpublished data); (4) the absence of neutralization activity of anti-IX serum towards homologous adenovirus (P. Boulanger & W. C. Russell, unpublished data).

A recent study has shown the involvement of tryptophan residues in the maintenance of the cohesion of the group of nine hexons (Boulanger et al. 1978b). These critical tryptophan residues could belong to the hexon and/or to IX, but it seems unlikely that the unique tryptophan in polypeptide IX could play this role while retaining the threefold symmetry. On the other hand the amount of tryptophan (1.2 to 1.5%) in the hexon protein (Boulanger & Puvion, 1973) is more consistent with a part in this cohesive process. It is also interesting that a viable deletion mutant of adenovirus has been obtained which produces virions devoid of polypeptide IX which are, however, significantly more heat sensitive than wild type virions (N. Jones, W. Colby and T. Shenk, personal communication). This suggests that IX is not indispensable for hexon bonding in groups of nine but is perhaps required for stabilization of the capsid.

The present communication conclusively demonstrates the synthesis of polypeptide IX early in infection as also described by Persson et al. (1978). In our experiments the polypeptide could only be distinguished from a background of low mol. wt. cellular polypeptides by separation in a two-dimensional gel.

Interestingly, we were unable to demonstrate the synthesis of polypeptide IX by immunoprecipitation from cell extracts labelled at early times in infection (Fig. 8) although polypeptide IX could be immunoprecipitated and analysed on two-dimensional gels from either type 2 or type 5 infected extracts prepared after prolonged incubation of cells with cytosine arabinoside. This 'early' pattern obtained by treatment with inhibitor may not be quite
equivalent to that obtained by pulse-labelling at early times in infection. Our findings may be explained by proposing that polypeptide IX may undergo maturation to attain an active antigenic conformation and it is possible that some of these various configurations may possess functions other than structural ones. Adenovirus polypeptide IX also provides an interesting example of a structural protein whose biosynthesis is regulated by a mechanism apparently different from that of other structural peptides, presumably at the level of transcription.

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REFERENCES


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