Virus-specific Proteins in Adenovirus Type 12-transformed and Tumour Cells as Detected by Immunoprecipitation

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(Accepted 25 November 1981)

SUMMARY

Adenovirus type 12 (Ad12)-specific proteins were determined in nine well-characterized Ad12-transformed hamster cell lines and in Ad12-induced hamster and rat tumour lines by immunoprecipitation, gel electrophoresis and autoradiography. All cell lines expressed a 60K mol. wt. polypeptide. In several cell lines, the presence of a 58K mol. wt. protein could also be demonstrated by these techniques. Smaller Ad12-specific proteins could be detected only in the Ad12-transformed hamster cell lines HA12/7 and A2497-3, and in the Ad12-induced hamster tumour line CLAC3. The quality of the immunoprecipitation tests performed depended primarily on the sera used; their antibody titres varied widely. For at least one of the Ad12-transformed hamster cell lines (HA12/7), there was good agreement in the number and mol. wt. of Ad12-specific proteins detected by both immunoprecipitation and in vitro translation of hybrid selected cytoplasmic RNA. There was no clear-cut correlation between the number and the nature of Ad12-specific proteins and the way in which the cell lines or tumours were obtained. Cell lines with the least number of copies of Ad12 DNA persisting appeared to express the largest number of Ad12-specific proteins.

INTRODUCTION

In recent years, detailed analyses have been carried out in our laboratory on a large number of adenovirus type 12 (Ad12)-transformed cell lines, on Ad12-induced tumours, and on cell lines established from these tumours. These cell lines and tumours were all of rodent origin. In particular, we have concentrated on studies relating to the patterns of persistence and integration of the virus DNA in some 50 different cell lines and Ad12-induced tumours (Groneberg et al., 1977; Sutter et al., 1978; Doerfler et al., 1979; Stabel et al., 1980; Eick et al., 1980; Ibelgaufts et al., 1980; Kuhlmann & Doerfler, 1982; Deuring et al., 1981; Eick & Doerfler, 1982). We have also analysed the patterns of methylation of specific segments of the integrated Ad12 genomes in some of these cell lines, and have observed an inverse correlation between the overall levels of DNA methylation in specific virus DNA segments and the extent to which these segments are expressed as messenger RNAs (Sutter & Doerfler, 1979, 1980; Vardimon et al., 1980; Eick et al., 1980). Recently, we have shown that DNA methylation at specific sites of a cloned adenovirus gene leads to transcriptional inactivation of this gene (Vardimon et al., 1981, 1982).

In many of the Ad12-transformed hamster cell lines, and the Ad12-induced hamster and rat tumour lines, the early virus gene sections are expressed to varying degrees (Ortin et al., 1976; Ibelgaufts et al., 1980; Schirm & Doerfler, 1981). In only a few of the Ad12-induced
rat (Ibelgaufts et al., 1980) and hamster tumour lines (Schirm & Doerfler, 1981) has late Ad12-specific cytoplasmic RNA been detected as well.

Using the method of in vitro translation of Ad12 DNA-selected cytoplasmic RNA from Ad12-transformed hamster cell lines, we have initiated a systematic survey of virus gene functions actually expressed in the transformed and tumour cell lines under investigation (Esche et al., 1979). Ad12-specific proteins in transformed cells have also been studied by Wold et al. (1979), Shiroki et al. (1979) and van der Eb et al. (1979). With improved techniques, this research has recently been extended to several Ad2- and Ad12-transformed cell lines (Jochemsen et al., 1980; Esche, 1982; Esche & Siegmann, 1982). The technique of in vitro translation of hybrid-selected RNA is highly sensitive. However, it cannot be stated with certainty that all of the cytoplasmic RNAs retrievable from transformed and tumour cells are ultimately translated in these cell lines, although that notion appears to be likely.

Another useful approach towards the detection of Ad12-specific proteins in virus-transformed cells and virus-induced tumour cells is that of immunoprecipitating virus proteins from extracts of these cells. It has been shown previously (Esche et al., 1979; Schrier et al., 1979; Jochemsen et al., 1980) that this experimental scheme is highly dependent on the quality of the immune sera used. In general, such sera are raised in Ad12 tumour-bearing hamsters or by injecting into rabbits extracts of human cells productively infected with Ad12 and harvested early or late after infection. There are problems of relatively low sensitivity and high background precipitation of non-viral proteins associated with this approach. It is, therefore, mandatory to perform each experiment very carefully using non-transformed cells and normal hamster sera as controls.

In the present report, we have identified a set of Ad12-specific proteins by immunoprecipitation in several Ad12-transformed cell lines and in Ad12-induced tumour cell lines. The stringent criteria for virus specificity outlined above have been observed. The results obtained in these investigations agree well with those gleaned from in vitro translation experiments using hybrid-selected virus RNA from the same transformed and tumour cells.

METHODS

Cells and virus. In the studies described here the following cell lines were employed. The hamster line T637 originated from transformation of BHK21 cells with Ad12 (Strohl et al., 1970); the hamster lines HA12/7, A2497-2 and A2497-3 were derived from primary hamster embryo cells by transformation with Ad12 (zur Hausen, 1973; A. M. Lewis, Jr, personal communication). The B3 hamster cell line was a subclone of BHK21 cells. Line CLAC3 was established from an Ad12-induced tumour in an inbred hamster (CLAC, apricot) as described by Stabel et al. (1980). Similarly, lines H313 and H1213 stemmed from Ad12-induced tumours in Syrian golden hamsters (Han Aura) as outlined elsewhere (Kuhlmann & Doerfler, 1982). All these cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) (Bablanian et al., 1965) enriched with 5 to 10% foetal calf serum. The B3 hamster cells were grown in the same medium except that 10% horse serum was used. The rat brain tumour lines RBT12/3 and RBT12/15 were established from Ad12-induced tumours of neuroepithelial origin (Ibelgaufts et al., 1980). Rat embryo fibroblasts and the lines RBT12/3 and RBT12/15 were grown in DMEM supplemented with 5% foetal calf serum and 2% horse serum. Human adenovirus type 12, strain Huie, was grown in human KB cells growing as monolayer or suspension cultures in Eagle's medium (Eagle, 1959) supplemented with 10% calf serum as described previously (Doerfler, 1969).

Preparation and preadsorption of sera. Tumours were induced in hamsters by injecting CsCl-purified Ad12 into hamsters within 24 h after birth (for details, see Kuhlmann &
Adenovirus proteins in transformed and tumour cells

Doerfler, 1982). When the tumours had grown to a size of about 3 × 4 cm, 2 to 3 ml of blood were obtained by heart puncture. Serum was prepared by conventional methods and stored in small samples at −20 °C. Control sera from normal hamsters were prepared in the same way.

Extracts of Ad12-infected KB cells (approx. 100 p.f.u./cell) were prepared by ultrasonic treatment of cells either 9 h (KB·Ad12 early) or 49 h (KB·Ad12 late) post-infection. The extracts were clarified by low-speed centrifugation and were mixed 1:1 with Freund's adjuvant. Rabbits were immunized twice by injecting 1 ml of the mixture (corresponding to 5 × 10^6 infected cells) subcutaneously within a 4 week period. Ten days later, sera were prepared and tested by standard procedures (Ouchterlony, 1958).

For some experiments, antisera were preadsorbed to KB or B3 cell extracts or to B3 cells directly. Cell extracts were prepared as described below; 1 vol. extract was mixed with 2 vol. hamster or rabbit serum, the mixture was incubated at 4 °C for 2 to 4 h and then centrifuged and stored at −20 °C. Sera from tumour-bearing hamsters were adsorbed to B3 cells grown in monolayers. Prior to preadsorption, the cells were washed with cold phosphate-buffered saline (PBS), treated with −20 °C methanol for 5 to 10 min and washed again. Sera were diluted 1:10 with PBS and adsorbed to the methanol-fixed cells for at least 24 h at 4 °C. The procedure was repeated several times.

Labelling of cells and preparation of cell extracts. Ad12-transformed and tumour cells were labelled with [35S]methionine by growing cells for 24 h in DMEM containing about 1/10 the normal concentration of methionine, 5% foetal calf serum and 25 μCi [35S]methionine per ml (sp. act. 1200 Ci/mmol, Amersham-Buchler). The RBT12/3 and RBT12/15 cells and rat embryo fibroblasts were maintained in normal medium for 24 h and were then labelled for 24 h with 25 μCi [35S]methionine per ml. In some experiments, KB cells growing in monolayers were infected with 150 p.f.u./cell Ad12. After a 2 h adsorption period, the infected or mock-infected cells were labelled with 25 μCi [35S]methionine per ml in Eagle's medium containing 1/10 the concentration of methionine and supplemented with 5% newborn calf serum for 8 h (KB·Ad12 early). A portion of the Ad12-infected cells was labelled with [35S]methionine between 48 and 56 h post-infection (KB·Ad12 late).

To prepare cell extracts, radioactively labelled cells were extensively washed with PBS, and 3 × 10^6 cells were lysed for 15 min at 0 °C in 0-3 ml 0·15 M-NaCl, 0·02 M-tris-HCl pH 8, 1% Nonidet P40 (NP40) and 1 mm-phenylmethylsulphonyl fluoride (PMSF, Sigma) (Edvardsson et al., 1978). Extracts were clarified by centrifugation and stored at −80 °C.

Immunoprecipitations. The technique of Crawford et al. (1980) was used with some modifications. [35S]methionine-labelled cell extract (0·15 ml) was mixed with 0·3 ml NET buffer (0·15 M-NaCl, 5 mM-EDTA, 50 mM-tris–HCl pH 7·4, 0·02% NaN₃, 0·05% NP40, 0·25% gelatin and 0·2% bovine serum albumin) and 10 μl of serum from normal hamsters. This mixture was incubated at 4 °C for 16 to 24 h, 50 μl of a 10% suspension of Staphylococcus aureus was added and incubation was continued on ice for 15 min. The mixture was centrifuged, and 10 μl of serum from tumour-bearing hamsters, of immunized rabbit serum or of control serum was added. The samples were incubated on ice for 1 h and 30 μl of a 10% suspension of S. aureus was added. Pansorbin (Calbiochem) was used and washed twice with NET buffer prior to application. After 15 min on ice, the bacteria were pelleted and washed once with NET buffer, then once with NET buffer containing 0·5 M-LiCl, and finally, twice with NET buffer. The complexes were released from the staphylococci by incubation (1 h at room temperature) in 50 μl 0·05 M-tris–HCl pH 6·8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 1·5 mM-PMSF. The bacteria were removed by centrifugation.

Analysis by polyacrylamide gel electrophoresis (PAGE). The immunoprecipitated [35S]methionine-labelled proteins were analysed by PAGE on SDS–12·5% polyacrylamide
gels following the procedures of Laemmli (1970) and Esche et al. (1979). On completion of electrophoresis, the proteins were fixed in the gel by treating for 16 to 24 h in 10% trichloroacetic acid, 10% acetic acid and 30% methanol. The gel was placed in Enhance (New England Nuclear) for 1 h and then washed for 1 h in water. The gel was dried on to Whatman 3MM paper for 2 h on a gel drier and autoradiographed on XR-5 film at -80 °C. The following 14C-labelled mol. wt. markers were used in most experiments: myosin (200 000), phosphorylase b (92 000), bovine serum albumin (69 000), ovalbumin (46 000), carbonic acid anhydrase (30 000) and lysozyme (14 300). Mol. wt. of Ad12-specific proteins were calculated as described by Weber & Osborn (1969). The lowest mol. wt. proteins resolved by our gel system were between 10 000 and 15 000.

RESULTS AND DISCUSSION

In each of the Ad12-transformed hamster cell lines and the Ad12-induced hamster and rat tumour lines investigated, virus-specific proteins could be detected by immunoprecipitation (Fig. 1, 2). One of the main problems with the experimental approach chosen to detect Ad12-specific proteins in extracts of transformed cells was that immunoprecipitation brought down proteins other than virus proteins. These proteins might have had domains
Adenovirus proteins in transformed and tumour cells

cross-reacting with the virus-specific antibodies or, more likely, might have bound non-specifically to the immunoglobulin in the serum. Therefore, in all experiments, extracts of hamster or rat cells which were not transformed by Ad12 were also treated with antisera from tumour-bearing hamsters. Moreover, extracts of Ad12-transformed cells were incubated with normal hamster sera as controls. Any proteins precipitated in one of these control experiments were not considered specific and were therefore not included in the data presented in Table 1 which summarizes the Ad12-specific polypeptides detected in cell lines T637, HA12/7, A2497-2, A2497-3, CLAC3, RBT12/3, RBT12/15, H313 and H1213.

Some of the polypeptides precipitated by Ad12-specific sera were found in many cell lines investigated, the 60K protein being common to all cell lines. Other polypeptides were found in many different lines, e.g. the 50K/51K protein, or the 32K protein (Fig. 1, 2). In some lines, like cell lines T637, A2497-2 and H313, only one polypeptide, the 60K protein, could be unequivocally identified as virus-specific. In several cell lines, a 58K to 59K polypeptide could also be seen. In that sense, there were similarities among Ad12-specific polypeptides in transformed and tumour cell lines of different origins. It has been demonstrated previously that cell lines HA12/7, A2497-3 and CLAC3 contain considerably higher concentrations of Ad12-specific cytoplasmic RNA than cell line T637 (Schirm & Doerfler, 1981). To some degree these findings were also reflected in differences in the amounts of Ad12-specific proteins precipitable from extracts of these cells (Fig. 1, 2), and in the degree to which hybrid-selected RNA purified from some of these lines could be translated in vitro to virus-specific proteins (Esche & Siegmann, 1982). The size distribution of Ad12-specific proteins immunoprecipitated from extracts of Ad12-induced tumour cell lines (a) CLAC3, (b) RBT12/15, (c) H313 and (d) H1213. Experimental conditions were otherwise as described in Methods and legend to Fig. 1. The following sera were used. (a) Lane 1, serum from the Ad12 tumour-bearing hamster H1315; lane 2, serum from a normal hamster. (b) Lane 1, serum from the Ad12 tumour-bearing hamster Ad12-4; lanes 2 and 3, sera from rabbits injected with extracts of Ad12-infected cells harvested early (9 h) and late (49 h) post-infection respectively; lane 4, serum from a normal hamster. (c) Lane 1, serum from the Ad12 tumour-bearing hamster H212; lane 2, serum from a normal hamster. (d) Lane 1, serum from the Ad12 tumour-bearing hamster H1313; lane 2, serum from a normal hamster; lane 3, ¹⁴C-labelled marker proteins were electrophoresed (no immunoprecipitation was performed).
Fig. 3. Control experiments using extracts of Ad12-infected or mock-infected KB cells. (a to d) Human KB cells were infected with Ad12 and extracts prepared 56 h post-infection as described in Methods. (e, f) Extracts of mock-infected KB cells were also analysed. The following sera were used for immunoprecipitation. (a) Hamster serum raised against T637 cells; (b, c) two different hamster sera raised against Ad12; (d) preimmune hamster serum; (e) serum as in (c); (f) serum as in (d). All other experimental details are as described in Methods.

proteins immunoprecipitable from extracts of transformed cells corresponded in general to that of Ad12-specific early proteins in extracts of Ad12-infected human cells. The data presented in Fig. 3 demonstrate that hamster sera against T637 cells or two different sera against Ad12 readily precipitate Ad12-specific proteins from extracts of Ad12-infected KB cells which were harvested 56 h post-infection (lanes a to c). Preimmune serum did not lead to specific immunoprecipitation (lane d). Neither specific serum (lane e) nor preimmune serum (lane f) precipitated specific proteins from mock-infected KB cells.

Fig. 2(b) shows the results of an experiment which compared the Ad12-specific polypeptides immunoprecipitable with the serum from an Ad12 tumour-bearing hamster (lane 1) with those precipitated by rabbit sera raised against extracts of productively infected
Adenovirus proteins in transformed and tumour cells

Table 1. Survey of Adl2-specific polypeptides detected in cytoplasmic extracts of cell lines by immunoprecipitation

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Adl2-specific polypeptides† found in cell line:</th>
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<tbody>
<tr>
<td>HA12/7</td>
<td>60K, 57K-59K, 51K, 36K, 34K, 32K, 31K, 24K, 19K</td>
</tr>
<tr>
<td>H37</td>
<td>47K‡, 46K‡, 39K‡, 36K, 34K, 32K, 31K, 24K, 19K</td>
</tr>
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</table>

* Details of the experimental procedures employed and the characteristics of the cell lines analysed have been described in the text.

† The Adl2-specific polypeptides are listed according to mol. wt. (×10⁻³). All mol. wt. determinations are approximations; their accuracy rests on the precision of the mol. wt. determined for the marker proteins used in the analysis.

‡ The two polypeptides 47K and 39K could be precipitated only with rabbit sera raised against KB·Adl2 early and Kb·Adl2 late extracts respectively (for details see text).

cells harvested 9 h and 49 h post-infection (lanes 2 and 3 respectively). The late rabbit serum precipitated an additional 39K polypeptide from extracts of RBT12/15 cells; early serum precipitated a 47K polypeptide. Other differences were not noted. As a control, serum from an Adl2 tumour-bearing hamster or serum from immunized rabbits was incubated with extracts from untransformed rat embryo cells. Adl2-specific proteins were not precipitated (data not shown). The summary of the protein data (Table 1) includes minor bands found in some of the cell lines. These minor bands were not always apparent (Fig. 1 and 2) but were observed in experiments not presented here.

Considerable variation was observed amongst the sera (derived from 24 different animals) in the ability to precipitate individual Adl2-specific proteins from cell extracts (data not shown). Similar differences in the potencies of sera were reported by Jochemsen et al. (1980). These differences might be due to individual variations in the production of antibodies among different animals. However, variations might also depend on the tumour, the stage of its development or to what extent intracellular Adl2-specific proteins are liberated and become recognizable by the immune system in different animals.

Recently, Esche & Siegmann (1982) have investigated the Adl2-specific proteins in the Adl2-transformed hamster line HA12/7 by in vitro translation of cytoplasmic RNA in a reticulocyte system and by analysing the Adl2-specific proteins by gel electrophoresis. A comparison of the data obtained by this method and the results accumulated by direct immunoprecipitation from extracts of HA12/7 cells are presented in Table 2. At least for this cell line, there was surprisingly good agreement between both sets of data, although in a few instances certain polypeptides were detected by one and not the other method. It should be
Table 2. Comparison of Ad12-specific proteins detected by direct immunoprecipitation from extracts or by in vitro translation of cytoplasmic RNA isolated from HA12/7 cells

<table>
<thead>
<tr>
<th>Region of A12 genome</th>
<th>E1B</th>
<th>–</th>
<th>–</th>
<th>E1A</th>
<th>E1</th>
<th>–</th>
<th>E1A</th>
<th>E1A</th>
<th>E4</th>
<th>E4</th>
<th>E1B</th>
<th>E4</th>
<th>E1B</th>
<th>–</th>
<th>E4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct immunoprecipitation†</td>
<td>60K</td>
<td>57K–59K</td>
<td>51K</td>
<td>36K</td>
<td>34K</td>
<td>32K</td>
<td>31K</td>
<td>–</td>
<td>24K</td>
<td>–</td>
<td>19K</td>
<td>(18K)</td>
<td>17K</td>
<td>15K</td>
<td>14K</td>
</tr>
</tbody>
</table>

* These data were taken from Esche & Siegmann (1982). 59K refers to a polypeptide of an apparent mol. wt. of 59 000.
† These values are those presented in Table 1. The assignment of the derivation of these polypeptides from specific parts of the virus genome is tentative and rests solely on size comparisons.
Adenovirus proteins in transformed and tumour cells 365

noted that identical mol. wt. of polypeptides obtained by either method do not prove their molecular identity.

It was concluded that by direct immunoprecipitation using selected sera from tumour-bearing hamsters, a portion of the virus-specific proteins present in extracts of Ad12-transformed cells or of Ad12-induced tumour cells could be precipitated. In particular, some of the lower mol. wt. polypeptides, however, remained undetected by this approach. The one Ad12-specific polypeptide that was brought down in the extracts of almost all the cell lines and by most of the sera used was the 60K protein. Thus, direct immunoprecipitation of virus-specific proteins from cellular extracts appears a useful method to demonstrate the presence of some virus proteins. This method will not allow the detection of all virus-specific proteins, particularly when they are present in relatively small amounts. The results observed yielded no obvious correlations between the way in which these lines were transformed or the biological properties of the cells to the specific array of Ad12-specific proteins detectable in any of these lines. An inverse correlation found, that might be worth mentioning, is between the number of Ad12 DNA copies integrated in a given cell line and the amount of Ad12-specific RNA detectable by blot hybridization experiments (Schirm & Doerfler, 1981) or the amount of Ad12-specific polypeptides (this study). Cell lines HA12/7 and CLAC3 contain 3 or 4 and 5 genome equivalents of Ad12 DNA respectively. Line T637, however, carries 22 copies of integrated Ad12 DNA, and yet the expression of the Ad12 genome is more extensive in lines HA12/7 and CLAC3. These data indicate that in some multicopy cell lines expression of virus DNA is very limited.

We want to thank Ingrid Kuhlmann for help with the animal experiments, Ute Brüggemann for preparing media and cells, and Gertrud Deutschländer for typing this manuscript. This research was supported by the Deutsche Forschungsgemeinschaft through SFB 74.

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(Received 28 September 1981)