REVIEW ARTICLE

The structure and functions of the adenovirus early region 1 proteins

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1. INTRODUCTION

Although the original identification of adenoviruses was as a result of clinical investigation (Rowe *et al.*, 1953) their real importance has been as tools for fundamental scientific research. They have proved to be of outstanding value in two major areas of interest—firstly, as defined biological agents capable of causing cellular transformation *in vitro* and oncogenesis *in vivo* and, secondly, as model systems in the study of the structure of the eukaryote genome and the way in which expression of individual genes is controlled. The suitability of adenoviruses for use in the study of both tumourigenicity and transformation became apparent after the recognition that certain of the virus serotypes [adenovirus 12 (Ad 12) initially] could cause tumours when injected into newborn rodents (Trentin *et al.*, 1962; Huebner *et al.*, 1962) and that cells grown in culture could be morphologically transformed by the virus (McBride & Wiener, 1964; Freeman *et al.*, 1967).

The many adenovirus serotypes have been divided into a number of groups, initially on the basis of immunological criteria and latterly on the basis of DNA restriction mapping studies. In this Review I shall concentrate almost exclusively on data accrued from the most studied members of two of these groups: Ad 12 (a representative of the highly oncogenic group A adenoviruses) and Ad 2 and Ad 5 (representatives of the non-oncogenic group C adenoviruses) (a complete classification is given in Gallimore *et al.*, 1985a). Obviously it is not possible to cover all aspects of adenovirus biology in a short report such as this; therefore the emphasis has been placed squarely on a biochemical consideration of the latest additions which had been made to our knowledge of the structure and functions of the adenovirus early region 1 (E1) proteins. Other aspects of the subject have been treated only briefly. Readers in need of more information on these are referred to recently published monographs (Ginsberg, 1984; Doerfler, 1984a,b,c, 1986) and review articles (Pettersson & Akusjarvi, 1983; Bernards & van der Eb, 1984; Branton *et al.*, 1985; Gallimore *et al.*, 1985a).

2. STRUCTURE OF THE ADENOVIRUS GENOME

The adenovirus genetic information is encoded by a double-stranded linear DNA molecule of M_r about $(2.0\text{--}2.4) \times 10^7$, depending upon virus serotype (van der Eb & van Kesteren, 1966; Green *et al.*, 1967). The DNA

comprises 34000–36000 base pairs and is transcribed in both the leftward and rightward directions. Individual genes have been mapped on the genome by using a variety of techniques—for example the genetic mapping of mutants, and translation of mRNAs selected by hybridization to specific DNA sequences—and these have recently been confirmed by detailed nucleotide sequence analysis [see Sussenbach (1984) and van Ormondt & Galibert (1984) for reviews, and Roberts *et al.* (1986) for the complete nucleotide sequence of the Ad 2 genome]. Fig. 1 presents, in summary form, the major mRNAs transcribed from the Ad 2 genome although the general features are similar for all serotypes. The prefixes ‘E’ and ‘L’ refer to early and late messages which are defined as those transcribed before and after the onset of viral replication, respectively. The major polypeptides translated from the mRNAs are also indicated.

The first adenovirus genes to be transcribed and expressed after the onset of viral infection have been designated E1 and comprise that region at the left-hand end of the DNA (r-strand). The organisation of that part of the genome is very similar in the four virus serotypes for which nucleotide sequence data is available (Ad 2, Ad 5, Ad 7 and Ad 12) and is summarized in Fig. 2. The E1 region between 1.3 and 11.2 map units has been subdivided into two major transcriptional units, E1A and E1B (Berk & Sharp, 1978; Chow *et al.*, 1979; Perricaudet *et al.*, 1979; Wilson *et al.*, 1979).

(a) Early region 1A (E1A)

The E1A polypeptides are the first to be expressed and rely on the host cell transcription and translation mechanism, except insofar as expression appears to be, to some extent, autoregulated [see section 5b(i)]. The E1A regions of all the virus serotypes which have been characterized are transcribed into 13 S (1.1 kb), 12 S (0.9 kb) and 9 S (0.6 kb) mRNAs, the latter of which is preferentially expressed late in infection. The 13 S and 12 S mRNAs are co-terminal at both the 3’ and 5’ ends, but differ in the extent of internal splicing (Berk & Sharp, 1978; Chow *et al.*, 1979; Perricaudet *et al.*, 1979). Since they are in the same reading frame most of their amino acid sequences are in common except for the additional internal peptides in the proteins encoded by the 13 S mRNA (an extra 46 residues for Ad 2 and 5 and an extra 31 residues for Ad 7 and Ad 12). The E1A proteins of Ad 5, Ad 7 and Ad 12 comprise 289 and 243, 262 and 231 and 266 and 235 amino acids respectively (see section 4). (Since there is little or no agreement on the size of the

Abbreviations used: Ad, adenovirus; E1, early region 1; kb, kilobases; BRKs, baby rat kidney cells; aa, amino acid(s); MHC, major histocompatibility, HERs, human embryo retinal cells; HSP, heat shock protein.

Fig. 1. Diagrammatic representation of the adenovirus transcription map

The double-stranded DNA molecule is arbitrarily divided into 100 map units and is transcribed in both the rightward (r) and leftward (l) directions. The locations and directions of the major early (E) and late (L) transcription units are shown. The splicing patterns of the mRNAs are not shown. The structural proteins IX and IVa2 are expressed mainly at late times in infection.

adenovirus E1 proteins as determined by SDS/polyacrylamide-gel electrophoresis they will be referred to by their amino acid number in the remainder of this article.)

(b) Early region 1B (E1B)

Two major products are transcribed from the E1B region early in infection, a 13 S mRNA (1.0 kb) and 22 S mRNA (2.2 kb) (Bos *et al.*, 1981). A third mRNA is transcribed late in infection and codes for a structural polypeptide, protein IX. Both 22 S and 13 S mRNAs share the same 5' and 3' sequences, but differ in the number of nucleotides which are spliced out. Two major polypeptides have been identified from the E1B region (496 and 175 aa in Ad 2, 482 and 163 aa in Ad 12), both of which may be encoded by the 22 S mRNA depending on which of two initiation codons serves as the start site. It should be noted, however, that they are translated in different reading frames and therefore have no amino acid sequence in common. The smaller protein may also be encoded by the 13 S mRNA. A third minor E1B protein has been observed which shares its *N*- and *C*-terminal amino acid sequences with the larger E1B polypeptide (Dijkema *et al.*, 1982; Virtanen *et al.*, 1982; Anderson *et al.*, 1984; Virtanen & Pettersson, 1985).

3. ADENOVIRUS E1 PROTEINS REQUIRED FOR TRANSFORMATION *IN VITRO*

Using calcium phosphate transfection (Graham & van der Eb, 1973) of adenovirus DNA it has been possible to show that rodent and human cells in culture can be completely transformed with DNA comprising only the E1 region (Graham & van der Eb, 1973; Shiroki *et al.*, 1977; van der Eb *et al.*, 1977, 1979; Sekikawa *et al.*, 1978; Dijkema *et al.*, 1979; Mak *et al.*, 1979; Byrd *et al.*, 1982;

Jochimsen *et al.*, 1982; Rowe *et al.*, 1984; Gallimore *et al.*, 1985b). Transformation of baby rat kidney cells (BRKs) has also been achieved with Ad 5 and Ad 12 DNA fragments encoding the E1A proteins and the smaller E1B proteins (van der Eb *et al.*, 1979; Jochimsen *et al.*, 1982; Gallimore *et al.*, 1985b) and with Ad 5 and Ad 12 DNA fragments encoding only the E1A proteins (van der Eb *et al.*, 1979; Houweling *et al.*, 1980; Gallimore *et al.*, 1985b). However, in most of these latter cases transformation occurred at a reduced frequency compared with that obtained with the analogous whole E1 region.

The characteristics of the different transformants vary somewhat depending on which DNA fragment is used for transformation. It has been concluded that the expression of all of the E1 proteins is required for 'full transformation'. Those cell lines transformed with only E1A DNA have been considered 'incomplete transformants' with differences in morphology (more fibroblastic in appearance), growth patterns and tumourigenicity (Houweling *et al.*, 1980; Gallimore *et al.*, 1985b). Although the E1B proteins seem to be required for complete transformation, in the absence of E1A they have no obvious morphological effect (van den Elsen *et al.*, 1983a).

Analysis of the transformation studies has allowed a number of conclusions to be drawn: (i) expression of both major E1A polypeptides is required for 'full transformation' (Montell *et al.*, 1984); (ii) in Ad 2/Ad 5 transformants expression of either one of the E1B proteins in addition to E1A is sufficient for complete transformation, whereas with Ad 12 both the 163 aa and 482 aa polypeptides are required (van der Eb *et al.*, 1979; Houweling *et al.*, 1980; Jochimsen *et al.*, 1982; van den Elsen *et al.*, 1983b; Gallimore *et al.*, 1985b); (iii)

Fig. 2. Structure of the E1 region of Ad 12(a) and Ad 5(b)

The positions of the promoters, poly(A) signals, splice donor and acceptor sites are shown. Sequences spliced out of the mRNAs are indicated by short broken lines (---). The sizes of mRNAs and proteins encoded by them are shown, E1A products on the left and E1B products on the right. Locations of the open reading frames from which the proteins are translated are indicated by boxes. Long broken lines (----) connect the coding regions in different reading frames which are joined in the mRNAs. The E1B mRNAs have the potential to direct the synthesis of more than one protein and the predicted coding regions for all these polypeptides are shown. $\circ$ indicates an initiation codon and | a termination codon (diagram courtesy of Dr P. Byrd).

transformation frequency is highest when DNA covering the whole of the E1 region is used (van der Eb *et al.*, 1979; Houweling *et al.*, 1980; Jochimsen *et al.*, 1982; Gallimore *et al.*, 1985b); (iv) as a corollary of the transformation studies it has been found that the tumorigenicity of established cell lines generated by restriction fragments which do not encompass the whole of E1 is reduced, with those cell lines that can produce tumours doing so with lower frequency and with extended latent periods (Gallimore *et al.*, 1985b).

On the basis of transformation studies, it has been suggested that the E1A region acts as an immortalizing gene such that it alters cellular metabolism so as to initiate transformation and confer immortality without producing a fully transformed phenotype. Expression of the E1B proteins is then required for full transformation. There can be little doubt, however, that this is a simplification of events, and it is likely that spontaneous genetic changes occur within the cell, which have a strong influence on the final transformed phenotype.

Adenovirus-transformed cells, when grown in culture, normally express those E1 proteins appropriate to the region of the viral genome which has been transfected and it has generally been found that detectable levels of the E1 polypeptides in both rodent and human transformants stay constant during prolonged tissue culture. It has also become apparent that this level of protein expression is strictly defined regardless of the number of transfected gene copies in the transformed cells (Whittaker *et al.*, 1984; Gallimore *et al.*, 1985b). Thus the steady-state levels of E1A and E1B polypeptides are very similar in a large number of rodent and human Ad 12 transformants. Also the level of E1A proteins is invariant irrespective of whether the cells have been transformed with either E1A DNA alone or the whole of the E1 region (Gallimore *et al.*, 1985b). It seems probable therefore that a minimal level of viral protein is required for transformation to occur, but over-expression results in cell death.

The roles of the adenovirus E1 proteins in determining the transformed phenotype have been studied with a wide variety of mutant viruses. Limitations of space preclude a detailed discussion of adenovirus mutants, but current literature has recently been reviewed in detail by Shenk & Williams (1984) and Young *et al.* (1985). A number of broad conclusions based on extensive studies can, however, be drawn: firstly, Ad 5 E1A (complementation group I) mutants generally fail to transform rat embryo cells, but produce abnormal transformants when infected onto BRKs at high dose; secondly, both major E1A polypeptides are necessary for full transformation (Montell *et al.*, 1982, 1984); thirdly, all of the E1B (complementation group II) mutants transform poorly (Ho *et al.*, 1982). Similarly, those Ad 12 E1B mutants which have been mapped to the 483 aa polypeptide generally fail to transform whereas some of the Ad 12 E1A mutants can be used to generate transformed lines. It is apparent therefore that E1 proteins perform different functions during the transformation by virus and during transformation following DNA transfection. In particular both the E1A and larger E1B polypeptides are involved in initiation, with E1A also being involved in the maintenance of the transformed phenotype following virion-mediated transformation (Ho *et al.*, 1982).

4. STRUCTURE OF THE MAJOR ADENOVIRUS E1 PROTEINS

(a) Amino acid sequences

Nucleotide sequences of the DNA comprising early region 1 have been determined for four virus types: Ad 2 (Gingeras *et al.*, 1982), Ad 5 (van Ormondt *et al.*, 1978; Maat & van Ormondt, 1979; Maat *et al.*, 1980; Bos *et al.*, 1981), Ad 7 (Dijkema *et al.*, 1980, 1982) and Ad 12 (Perricaudet *et al.*, 1980; Fujinaga *et al.*, 1979; Sugisaki *et al.*, 1980; Kimura *et al.*, 1981; Bos *et al.*, 1981). From these, primary structures have been predicted for the two E1A polypeptides and the two major E1B proteins. Regions of homology in the proteins from the different virus serotypes are considerable, with about half of the residues in common (precise numerical values are given by the difference matrices shown in Table 1).

To give maximum homology it is necessary to introduce a limited number of deletions into the E1B sequences, but rather more into E1A. In particular, it appears that appreciable numbers of nucleotides have been lost from the Ad 7 and Ad 12 genes encoding amino acids from residues 110–122 and 212–246. A further major 20 amino acid deletion must be introduced into the Ad 2/5 E1A sequence from residues 146 to 166 to allow alignment of the following amino acids.

Table 1. Difference matrices for the amino acid sequences of the adenovirus E1 proteins

The numbers shown are percentage differences between sequences counting deletions as an extra amino acid. The values in parentheses in section (a) are difference matrices for the E1A 12 S message, those outside parentheses are for the 13 S message.

	Ad 2	Ad 5	Ad 7	Ad 12
(a) E1A protein				
Ad 2	0 (0)	2 (2)	70 (75)	66 (70)
[289 (243) aa]				
Ad 5	2 (2)	0 (0)	70 (74)	66 (70)
[289 (243) aa]				
Ad 7	70 (75)	70 (74)	0 (0)	57 (57)
[262 (231) aa]				
Ad 12	66 (70)	66 (70)	57 (57)	0 (0)
[266 (235) aa]				
(b) Smaller E1B protein				
Ad 2	0	2	51	62
(175 aa)				
Ad 5	2	0	50	61
(174 aa)				
Ad 7	51	50	0	56
(178 aa)				
Ad 12	62	61	56	0
(163 aa)				
(c) Larger E1B protein				
Ad 2	0	2	47	53
(496 aa)				
Ad 5	2	0	47	53
(496 aa)				
Ad 7	47	47	0	54
(492 aa)				
Ad 12	53	53	54	0
(482 aa)				

(i) **E1A proteins.** All of the E1A polypeptides are very acidic with isoelectric points between pH 4 and 5. Most of the acidic residues, however, are grouped in the *N*-terminal halves of the proteins (for example, for the Ad 12 266 aa polypeptide the net charge for the *N*-terminal 133 amino acids is -31 whereas for 134–266 it is only -5). Amino acids conserved between the virus serotypes tend to be grouped to give areas of homology (for example, residues 41–89, 123–144, 166–211, which includes the regions unique to the 13 S message, and 246–260). In these areas amino acid substitutions are generally of a conservative nature. Presumably functional constraints have been placed on these homologous regions whereas areas which have unique structures are involved in those properties of the proteins which are restricted to one virus serotype [e.g. the regulation of expression of MHC class I antigen; see section 5*b*(ii)]. The amino acids present in the sequence unique to the larger E1A polypeptide are highly conserved between Ad 2/5, Ad 7 and Ad 12 and they are obviously important in conferring on the larger proteins those functions which cannot be fulfilled by the smaller polypeptides (see section 5 and Glenn & Ricciardi, 1985).

The M_r values of the E1A proteins determined from the nucleotide sequences are appreciably less than those observed after SDS/polyacrylamide-gel electrophoresis. For example, calculated values for the Ad 12 266 aa and 235 aa polypeptides are 29658 and 26021 respectively (Sugisaki *et al.*, 1980) whereas the major E1A proteins have observed M_r values in excess of 40000. It has been suggested that this discrepancy is due to the high proline content, but on this basis it might be expected that there would be a much greater difference between the observed and calculated M_r values for the Ad 2/5 proteins, in which proline accounts for 16% of the amino acids, than for the Ad 12 proteins, in which only 9% of the residues are proline. In fact, this is not really the case. Whereas the high proline content may be one factor influencing protein behaviour on polyacrylamide gels, it is possible that there may be incomplete denaturation under the electrophoresis conditions used, causing the polypeptides to migrate anomalously.

A comparison of the amino acid sequences of E1A polypeptides from different virus serotypes reveals expected regions of similarity. It has also been suggested that the E1A proteins are homologous to the *myc* and *myb* oncogenes (Ralston & Bishop, 1983). However, using a rigorous statistical treatment McLachlan & Boswell (1985) have discounted these latter claims and concluded that there is no ancestral relationship between the adenovirus proteins and the *c-myc* oncogene.

(ii) **E1B proteins.** No large deletions have to be introduced into the E1B sequences to maximize the homology although it appears that a limited number of nucleotides have been lost. Both E1B proteins from the four virus serotypes are relatively highly conserved, but it is notable that the homologies are appreciably less marked at the *C*-termini of the smaller proteins (approx. residues 130–180) and at the *N*-termini of the larger proteins (approx. residues 1–100).

(b) Multiple forms of the E1A proteins

Initial investigations showed that a number of highly related protein species were encoded by E1A. Obviously two of these may be explained by the two major mRNAs (13 S and 12 S) transcribed, but it appears that each of

the 13 S and 12 S products can exist in a number of forms. Using anti-peptide sera Yee *et al.* (1983) and Yee & Branton (1985*a*) have confirmed that each mRNA is translated into at least two major and two minor products, all of which share the same *N*- and *C*-termini and can be phosphorylated to a comparable degree (Yee & Branton, 1985*a*). In a rather more detailed study using monoclonal antibodies and two-dimensional gel electrophoresis Harlow *et al.* (1985) identified 40–60 polypeptides originating from the E1A gene region. It is likely that a number of these species could be intermediates in major, as yet undefined, post-translational modifications (see section 4*c*), but even so it is still far from clear how or why the virus requires the large number of forms of the E1A proteins which do not fall into this category.

(c) Post-translational modifications of the E1 proteins

Two of the post-translational modifications commonly encountered in other systems have been shown to occur with the adenovirus E1 proteins, namely phosphorylation and acylation. It appears that all of the major forms of the Ad 5 and Ad 12 E1A proteins are phosphorylated at multiple sites (Yee *et al.*, 1983; Rowe *et al.*, 1983*a*; Scott *et al.*, 1984; Lucher *et al.*, 1985). Serine is the only phosphorylated amino acid which has been detected in the native Ad 12 E1A polypeptides or in E1A proteins synthesized using expression vectors and then phosphorylated *in vitro* (Lucher *et al.*, 1985). Initial investigations have shown that the Ad 12 E1A 266 aa and 235 aa proteins produced in bacteria are phosphorylated by an extract of KB cells in a similar manner to the *in vivo* product. The protein kinases involved in this process appear to be relatively non-specific, phosphorylating many of the serine residues along the polypeptide chain and are Ca^{2+} and cyclic AMP-independent (Lucher *et al.*, 1985).

The E1A proteins also seem to be subject to a second post-translational modification which causes an apparent increase in M_r as determined by SDS/polyacrylamide-gel electrophoresis (Branton & Rowe, 1985; Fergusson *et al.*, 1985*b*). The site of this modification has been localized to that part of the protein encoded by the first exon (Fergusson *et al.*, 1985*b*), but as yet no more information is available. A common cause of apparent increases in the M_r values of proteins is glycosylation, but no carbohydrate moieties have been detected in covalent association with the Ad 12 E1A polypeptides (Grand *et al.*, 1985). Whether multi-site phosphorylation and the uncharacterized modification noted by Fergusson *et al.* (1985*b*) are sufficient to explain the multitude of E1A species detected by Harlow *et al.* (1985) (see section 4*b*) is doubtful; therefore, it is probable that other post-translational processing reactions will be revealed as our knowledge of the biochemistry of E1A increases. It is likely, however, that the apparently universal nature of the phosphorylation reactions involving E1A make it of considerable importance in the functioning of the protein during virus infection and may be a way in which its mode of action is subtly regulated.

The larger adenovirus E1B protein is also phosphorylated in Ad 5 infected cells (Levinson & Levine, 1977; Malette *et al.*, 1983). Two residues of serine and one of threonine have been identified as phosphate acceptors (Malette *et al.*, 1983), but at present little more information is available.

The Ad 12 E1B 163 aa protein contains covalently bound lipid which is probably of importance in its localization in the cell membrane (Grand *et al.*, 1985) as discussed in section 5(a). The acyl group is attached to the protein through an ester linkage, probably via a serine or threonine residue in the amino acid backbone (Grand *et al.*, 1985). Preliminary evidence suggests that a similar modification also occurs with the equivalent Ad 5 protein (Branton *et al.*, 1985).

(d) Stability and half-life of the E1 proteins

Using [³⁵S]methionine labelling and pulse-chase techniques the half-lives of the Ad 5 E1 proteins have been determined in infected and transformed human cells (Spindler & Berk, 1984; Branton & Rowe, 1985). The 176 aa and 496 aa E1B polypeptides are relatively stable in infected cells with only about 30% being turned over during a 4 h chase period (Branton & Rowe, 1985). The E1A polypeptides, however, are much more labile with calculated half-lives for the 289 aa and 243 aa polypeptides of 35–55 min and 80–90 min respectively in infected and transformed cells (Spindler & Berk, 1984; Branton & Rowe, 1985). The minor, lower-*M_r* species of each E1A polypeptide is even less stable, adding further weight to the argument that it is a quickly modified intermediate in the synthesis of the final protein product (Branton & Rowe, 1985).

(e) The use of expression vectors in studies on E1 proteins

Direct purification and characterization studies on adenovirus E1 polypeptides are difficult for two major practical reasons: firstly, the proteins are present within both transformed and infected cells at very low concentrations and secondly, the physical problems involved in growing the numbers of cells which would constitute a reasonable amount of starting material for most biochemical experiments. To overcome some of these difficulties much use has been made in the past few years of expression vectors which allow the synthesis of large amounts of either partial or complete proteins in bacteria.

Six adenovirus E1 proteins have been expressed at high level and purified to homogeneity after transfection of plasmid expression vectors into *Escherichia coli*: the Ad 2/5 E1A 289 aa protein (Fergusson *et al.*, 1984; Krippl *et al.*, 1984), the Ad 2/5 E1A 243 aa protein (Fergusson *et al.*, 1985a), the Ad 12 E1A 266 aa and Ad 12 E1A 235 aa proteins (Kimelman *et al.*, 1984), the Ad 2 E1A 289 aa protein and the Ad 2 E1B 496 aa protein (Ko & Harter, 1984). In most cases the adenovirus proteins are expressed as several per cent of the total cellular protein after induction and are very similar to the major polypeptides produced during viral infection.

A drawback of the method is that relatively stringent conditions have to be used to solubilize the viral protein during purification (e.g. exposure to 2M-potassium thiocyanate; Krippl *et al.*, 1984) and it might be considered that this would result in denaturation and partial loss of biological activity. However, it has been shown that the Ad 2/5 E1A polypeptide can localize to the nucleus of microinjected cells and is capable of activating viral gene expression (Krippl *et al.*, 1984; Fergusson *et al.*, 1985a,b). One major disadvantage of the prokaryote expression system is that most of the post-translational modifications normally encountered

in eukaryotic cells do not take place. Therefore, the protein products of expression vectors tend to be unmodified polypeptide chains. Fergusson *et al.*, (1985a,b) have noted that E1A polypeptides become modified when injected into a number of different eukaryote cell types, as evidenced by an apparent increase in *M_r* following SDS/polyacrylamide-gel electrophoresis. It is probable that this represents a post-translational modification of some type, which is important for the correct functioning of the protein (Fergusson *et al.*, 1985b). Obviously, if the modification is of importance for biological activity and does not occur automatically (as does the one observed by Fergusson *et al.*, 1985b) this could lead to erroneous results in future characterization experiments. The E1A polypeptides purified from bacteria do not contain covalently bound phosphate groups as do the *bona fide* proteins, but incubation with a crude kinase preparation, made from human KB cells, results in a phosphorylation pattern very similar to that found in infected cells (Lucher *et al.*, 1985).

Bacterial expression systems have also been used to generate, in high yield, fusion proteins encoded by parts of one of the adenovirus E1 genes covalently linked to a bacterial gene (e.g. *trpE* or β -galactosidase). The fusion proteins have generally been used to produce specific high-titre antibodies (Spindler *et al.*, 1984; Scott *et al.*, 1984; Harlow *et al.*, 1985). Harlow *et al.* (1985) have used mice injected with an Ad 2 E1A-*trpE* fusion protein to make a panel of monoclonal antibodies which have been used in the characterization of multiple forms of the E1A polypeptides present in infected cells (see section 4c). These fusion protein antisera provide superior alternatives to tumour bearer sera which have usually been used in the past for the detection of the adenovirus E1 proteins. A third possibility has been to make antibodies directed against short synthetic peptides, the sequences of which are based on the determined nucleotide sequence of E1 (Yee *et al.*, 1983; Rowe *et al.*, 1983a; Green *et al.*, 1983a,b; Feldman & Nevins, 1983).

5. FUNCTIONS OF THE ADENOVIRUS E1 PROTEINS

As the power of molecular biology allows us to identify gene products of general biological significance the problem of deducing specific biochemical functions for particular peptides is one with which we are being faced more and more. This situation is well illustrated by the case of the adenovirus E1 proteins, which have been identified for several years, but about which we know relatively little. However, a number of approaches to the problem have been tried and these will be considered in detail in the following section.

(a) Subcellular localization of E1 proteins

Any protein must be located within the cell at its point of action; therefore, it was considered that an investigation of subcellular distribution might provide some functional clues. In subcellular fractionation experiments the E1A and the larger E1B proteins were found to be evenly distributed between the nucleus and cytoplasm in Ad 5 infected KB cells (Rowe *et al.*, 1983b) and in Ad 12 transformed human and rat cells (Grand & Gallimore, 1984). The nuclear forms of the E1A polypeptides are associated with the nuclear matrix (Feldman & Nevins,

1983) or nucleoplasm (Rowe *et al.*, 1983b), but not with DNA (Fergusson *et al.*, 1985b). A portion of the cytoplasmic E1A proteins are bound to cytoskeletal structures in Ad 5 infected (Rowe *et al.*, 1983b) and Ad 12 transformed cells (Grand & Gallimore, 1984). The particular structures involved are probably microtubules and 10 nm filaments. Whether this localization is important in the morphological changes which accompany transformation by adenoviruses is not yet clear, but it is obvious that this is one point at which a direct influence may be exerted by the E1A polypeptides.

Immunofluorescence studies have provided confirmatory data and it has been observed that Ad 5 infected cells exhibit a strong nuclear fluorescence and diffuse cytoplasmic staining due to the presence of E1A proteins (Yee *et al.*, 1983; Feldman & Nevins, 1983) or to the larger E1B polypeptide (Sarnow *et al.*, 1982a; Yee *et al.*, 1983; White *et al.*, 1984). It has also been noted that when purified Ad 2/Ad 5 E1A polypeptides (equivalent to the products of the 12 S and 13 S mRNA) are microinjected into Vero cells they quickly localize in the nucleus where they are functionally active (Krippl *et al.*, 1984; Fergusson *et al.*, 1985a,b).

The location of the smaller E1B protein has been mapped with rather more precision (White *et al.*, 1984; White & Stillman, 1985). Indirect immunofluorescence studies have shown the Ad 2 polypeptide to be present, early in infection and in transformed cells, in the nuclear envelope and in the cytoplasm, possibly in the membranes of the endoplasmic reticulum. Late in infection, the E1B 175 aa protein is in the inner and outer nuclear envelopes with about half bound to the nuclear lamina (White *et al.*, 1984). In subcellular fractionation experiments the protein co-purifies with various membrane preparations from Ad 5 infected cells (Rowe *et al.*, 1983b) and Ad 12 transformed cells (Grand & Gallimore, 1984). It has also been purified to homogeneity from Ad 2 infected HeLa cell membranes (Persson *et al.*, 1982) and been shown to be present on the external surface of Ad 12 transformed human cells (Grand *et al.*, 1985). Localization of the protein in a number of different types of cellular membranes could be due to the covalent attachment of lipid moieties to the polypeptide chain directly after synthesis (Grand *et al.*, 1985; Branton *et al.*, 1985; see section 4d).

(b) E1 proteins as regulators of gene expression

Whilst our knowledge of early region 1 function is very limited, one characteristic has emerged over the past 8 years, and that is the ability of the E1A polypeptides to regulate the level of expression of both viral and cellular genes.

(i) Regulation of viral gene expression by E1A. Using a variety of Ad 5 mutants with lesions in the E1A gene it was originally shown that expression of the E1A gene product was required for the accumulation of early viral mRNAs encoded from regions E1B, E2, E3 and E4 (Jones & Shenk, 1979a,b; Berk *et al.*, 1979). Since that time our knowledge of how this process occurs has become more detailed, based largely on a number of sophisticated investigations employing the tools of molecular biology, but information at the basic biochemical level has not been forthcoming.

Before discussing its general role in regulation it should be noted that E1A is capable of autoregulation at

the level of transcription (Berk *et al.*, 1979; Nevins, 1981; Osborne *et al.*, 1984; Hearing & Shenk, 1985; Tibbetts *et al.*, 1986). Mutant studies have shown that the minimal upstream sequence required for the full up-regulation of transcription of E1A is the TATA box (Hearing & Shenk, 1985) and even when this is missing some activation still occurs (presumably with homologous upstream sequences being used in its place). It has also been suggested that the adenovirus E1A region is capable of negative autoregulation (Tibbetts *et al.*, 1986) such that E1A gene products may be able to down-regulate E1A transcription at times late in infection, possibly as part of the 'early to late switch'.

The E1A gene product has been specifically implicated in the up-regulation of expression of all of the adenovirus early gene regions: E1B (Rossini, 1983; Bos & ten Wolde-Kraamwinkel, 1983), E2 (Imperiale *et al.*, 1983; Rossini, 1983; Weeks & Jones, 1983; Leff *et al.*, 1984; Krippl *et al.*, 1984; Kingston *et al.*, 1984; Guilfoyle *et al.*, 1985), E3 (Weeks & Jones, 1983; Leff *et al.*, 1984; Courtois & Berk, 1984) and E4 (Weeks & Jones, 1983; Svensson & Akusjarvi, 1984). These reports agree on the fact that activation is through a *trans*-acting factor (although there may also be a *cis*-acting factor as well) which is diffusible and is almost certainly a polypeptide chain encoded by the E1A gene. The activation has been shown to occur through an increase in the rate of transcription of the target gene (Nevins, 1981; Weeks & Jones, 1983; Leff *et al.*, 1984). Whether both of the E1A major products can function equally well in this up-regulation is not clear. Carlock & Jones (1981), Leff *et al.*, (1984), Winberg & Shenk (1984) and Fergusson *et al.*, (1985a) have shown that stimulation of Ad 2 E2 or E3 genes may be accomplished as efficiently by the product of the 12 S mRNA as by the 13 S product. However, a number of other groups (Berk *et al.*, 1979; Ricciardi *et al.*, 1981; Bos & ten Wolde-Kraamwinkel, 1983; Montell *et al.*, 1984; Svensson & Akusjarvi, 1984) have found little activation by the smaller protein. Whilst it is rather difficult to reconcile these conflicting data, it seems probable that they may be attributed to basic differences in the assay systems employed.

It has been suggested that the two E1A proteins can function in concert to exert a rather more complex regulatory influence over the transcription of E2A (Rossini, 1983; Guilfoyle *et al.*, 1985). The larger polypeptide is essential for expression of the DNA-binding protein (encoded by E2A) when the gene is associated with its early promoter, whereas when the gene is associated with its late promoter only, expression is at a high level in the absence of other activating proteins. Addition of the smaller E1A protein in this case is then capable of inhibiting the expression.

Activation of viral early region genes by E1A occurs when the target gene is integrated into the cellular chromatin (Courtois & Berk, 1984; Kingston *et al.*, 1984) to a similar extent to when it is present as a free plasmid. Conversely, the up-regulation occurs when the target gene is transfected into 293 cells which constitutively express the Ad 5 E1A proteins (Imperiale *et al.*, 1983; Svensson & Akusjarvi, 1984).

It is reasonable to assume that there must be specific DNA sequences adjacent to the adenovirus early genes which make them receptive to activation by the E1A proteins, since the introduction of E1A DNA or

polypeptides does not result in a general up-regulation of all of the cellular genes present. It has been shown that DNA sequences present in the 250 nucleotides 5' to the initiation sites for E1B, E2A, E3 and E4 are required for activation (Weeks & Jones, 1983; Bos & ten Wolde-Kraamwinkel, 1983; Imperiale & Nevins, 1984). With the E2 gene, at least, the upstream regulatory sequence is activatable in either orientation and is capable of causing an increase in gene expression in response to E1A protein even if separated from the E2 gene by up to 4000 nucleotides, suggesting that it may possess enhancer-like properties. Whether this applies to all of the adenovirus early gene regulatory sequences is not yet clear. However, the nucleotide homology observed is poor and it seems probable that the required sequences may be some of those constituting the promoter element. The suggestion of Kingston *et al.*, (1984) that activation by E1A is, to some extent, determined by the fact that it is directed primarily against exogenously added DNA (by transfection, microinjection or infection) rather than by specific sequences may contain an element of truth, but it does not explain how adenovirus genes integrated into the host cell chromosome are also regulated.

All of the results detailed above are explicable on the basis of E1A gene products acting *in trans* (summarized in Fig. 3). There is, however, substantial evidence to suggest that activation by E1A may also occur in a *cis*-fashion. It has been recognized for some time that adenovirus mutants with lesions in E1A which fail to express the E1A polypeptides [for example, Ad 5 *dl* 312 (Jones & Shenk, 1979b) and Ad 12 *in* 601 (Gallimore *et al.*, 1986a)] eventually transcribe the products of the other early gene regions. It appears that this phenomenon may be explained on the basis of *cis* activation via a sequence of DNA probably upstream of the E1A initiation site (Gaynor & Berk, 1983; Hen *et al.*, 1983; Hearing & Shenk, 1983; Weeks & Jones, 1983; Imperiale *et al.*, 1983).

(ii) Regulation of cellular gene expression by E1A. Contemporary with the investigations into the control of virus gene expression by E1A there have been a number of studies carried out which conclusively demonstrate the ability of E1A to up-regulate cellular gene expression. Obviously this is not a generalized phenomenon, since the rate of transcription of cellular DNA does not increase following adenovirus infection (indeed it falls dramatically), but there are a limited but increasing number of genes which have been shown to be susceptible to E1A control.

During adenovirus 5 infection of HeLa cells there is an appreciable rise in the level of 70 kDa heat shock protein (HSP70) both at the protein and mRNA level (Nevins, 1982; Kao & Nevins, 1983); late in infection levels return to normal. Based on the results of mutant studies this phenomenon is directly attributable to effects of the E1A polypeptides on transcription of the cellular gene (Nevins, 1982; Kao & Nevins, 1983). Similar, although not so marked, increases have been observed with the host cell thymidine kinase (Ledinko, 1967; Braithwaite *et al.*, 1983), dihydrofolate reductase (Yoder *et al.*, 1983), and β -tubulin (Stein & Ziff, 1984) genes following Ad 2/5 infection. In other experiments where the target protein was encoded by a DNA plasmid transfected into human cells it was shown that activation of the β -globin gene occurred in response to the E1A protein provided either

by infection, transfection or constitutive expression (as in 293 cells) (Green *et al.*, 1983c). Similarly, the introduction of the rat preproinsulin I gene into a system where E1A could be expressed shows that the viral polypeptide can cause up-regulation (Gaynor *et al.*, 1984). However, it should be noted that increased transcription of neither the cellular β -globin nor preproinsulin I genes occurred in response to E1A (Stein & Ziff, 1984; Gaynor *et al.*, 1984).

Although the regulatory action of Ad 2/5 E1A on levels of expression of certain cellular genes in HeLa cells is now well established, it appears that Ad 12 E1A gene products do not always produce a similar effect. Indeed, Grand & Gallimore (1986) could detect no changes in HSP70 or β -tubulin, at the protein level, following Ad 12 infection of human embryo kidney cells. However, thymidine kinase activity was found to rapidly increase. A detailed study using a panel of Ad 12 mutants showed that the initial increase in expression of the thymidine kinase gene was attributable to the E1A 266 aa protein, but for continued gene expression one or more products of the E1B genes had to be present (Grand & Gallimore, 1986). These results contrast with those obtained following Ad 5 infection of rat cells (Braithwaite *et al.*, 1983) where the E1A gene product alone is sufficient to stimulate thymidine kinase gene expression.

As well as fulfilling a role as an activator of cellular gene expression the E1A proteins can also act as repressors of transcription. In an assay system containing the β -globin gene linked to SV40 or polyoma enhancers, E1A is capable of shutting down transcription by interaction with the enhancer element (Borrelli *et al.*, 1984). Similarly, in a transient expression assay both the Ad 5 289 aa and 243 aa proteins were found to be capable of repressing transcription (Velcich & Ziff, 1985). Whether these experiments indicate that the two major E1A products could function differently in regulation, with perhaps one acting positively and the other negatively, is not yet clear, but it remains an interesting possibility.

Almost all of the studies described above have been carried out using infected cells and on the basis of the results the authors have speculated as to how important the regulatory function of the E1A proteins might be in the acquisition and maintenance of the transformed phenotype. Despite this, studies of protein levels in adenovirus-transformed cells have been very rare (with the exception of MHC class I antigen—see below). HSP70 was shown to be present in large amounts in the Ad 5 transformed human cell line 293 (Nevins, 1982) and we have recently confirmed that in a large number of Ad 2/5 and Ad 12 transformed human embryo retinal cell lines there is an elevated level of expression of this protein compared with primary cells (Grand & Gallimore, 1986). Similarly, amounts of β -tubulin in these transformants are increased. However, there is considerable variation (up to 10-fold in the case of HSP70) between the different cell lines, regardless of the level of expression E1A, which is very similar in all of the lines examined. Whether the E1A region is of Ad 12, Ad 2 or Ad 5 origin appears to have little effect, except that there is a tendency for the Ad 2/5 transformants to express higher levels of HSP70 than their Ad 12 counterparts, whereas the converse applies to β -tubulin levels (Grand & Gallimore, 1986).

Probably of more significance are the recent studies

Fig. 3. Summary of the roles played by adenovirus E1 proteins in the regulation of the level of expression of viral and host proteins

Proteins are indicated by blocks and are arranged in the same orientation as the genes shown in Fig. 1. A stimulatory effect on the level of expression is indicated by a solid arrow and an inhibitory effect by a broken arrow. The point of the arrow indicates the affected polypeptide.

which have concentrated on the level of expression of the MHC class I antigen in adenovirus transformed rat cells. Schrier *et al.* (1983) demonstrated that the transformation of rat cells with Ad 12 led to a considerable reduction in detectable MHC on the surface compared with primary cells or those transformed with Ad 5. Further studies of a number of transformed cultures showed that the reduced expression of MHC in Ad 12 lines could be attributed to an active 'down-regulation' by the product of the Ad 12 E1A gene (Schrier *et al.*, 1983; Eager *et al.*, 1985; Vaessen *et al.*, 1986) and that this activity resided in the first exon (Jochimsen *et al.*, 1984). Ad 12 transformed rodent cells, with reduced expression of MHC on the surface, were found to be highly tumourigenic when injected into the syngeneic host (Tanaka *et al.*, 1985; Eager *et al.*, 1985) and it was confirmed that the cell lines derived from the tumours continued to express only low levels of MHC when grown in culture (Eager *et al.*, 1985). The tumourigenicity of Ad 12 transformed mouse cell lines could be reduced following introduction of MHC class I gene by transfection (Tanaka *et al.*, 1985) or by increasing the level of gene expression after treatment with interferon (Hayashi *et al.*, 1985). The obvious inference from these results is that the major difference in tumourigenicity between the group A (e.g. Ad 12) and group C (e.g. Ad 2 or Ad 5) adenoviruses is attributable to differences in the ability of E1A genes to down-regulate expression of MHC class I antigens.

The effects on MHC expression are not, however, achieved by short term exposure to the Ad 12 E1A gene products, since infection of primary cells with Ad 12 or Ad 12 mutants does not result in a reduction of mRNA (Rosenthal *et al.*, 1985). Indeed the level of mRNA from the H-2K^b transplanation antigen gene increases due to transcriptional activation (Rosenthal *et al.*, 1985). The situation becomes even more complex when Ad 12 and Ad 2/5 transformed human cell lines are examined. In a large number of Ad 12 E1 and E1A and Ad 5 E1 transformants we have found a marked reduction in the level of MHC class I antigen both on the surface and

within the cells (generally 5–10-fold), with only small differences between the two virus types (R. J. A. Grand, M. Rowe & P. H. Gallimore, unpublished results). An appreciable distinction, however, can be drawn when comparing transformants produced after transfection of an adenovirus E1A plasmid together with an activated N-ras plasmid (Gallimore *et al.*, 1986a,b). In those cell lines expressing Ad 12 E1A, MHC expression is similar to that found with the E1 transformants; however in those cell lines containing Ad 2 E1A, levels of MHC class I antigen are much more nearly normal (R. J. A. Grand, M. Rowe & P. H. Gallimore, unpublished results). It seems reasonable to conclude that in the human system, the presence of Ad 12 E1A alone will down-regulate MHC expression, whereas Ad 2/5 E1A causes a reduction only when present in conjunction with E1B.

Apart from exerting effects on individual protein expression, adenovirus infection causes fluctuations in cellular metabolism which result in considerable alterations in the growth cycle in non-permissive and semi-permissive cells (Braithwaite *et al.*, 1981, 1983). These changes can be attributed solely to the influence of the E1A gene products (Braithwaite *et al.*, 1983), and more specifically to the Ad 2/5 E1A 289 aa polypeptide (Bellet *et al.*, 1985).

On the basis of the observations that the expression level of a number of proteins which are cell-cycle-dependent are also stimulated by E1A gene products it has been suggested that a cellular E1A-like activity may be present which can control some aspects of cell-cycle-dependent transcription (Kao *et al.*, 1985; Liu *et al.*, 1985; Bellett *et al.*, 1985). Further evidence is provided for a cellular E1A-like activity by the observation that those lines which constitutively express high levels of HSP70, the expression of which is cell-cycle-dependent, more readily support the expression of early adenovirus genes, in the absence of E1A, following mutant virus infection (Imperiale *et al.*, 1984). Despite these interesting indications, considerably more rigorous evidence will have to be forthcoming before the idea of a functional similarity between viral and cellular genes can be

accepted. The ability of E1A defective mutants to express other early region gene products is greatly enhanced by treatment of the cells with protein inhibitors either immediately before or after infection (Katze *et al.*, 1981, 1983; Nevins, 1981). These data have been interpreted as evidence that E1A functions by binding to a cellular factor which inhibits or represses viral protein synthesis (Persson *et al.*, 1981).

In summary it can be stated that during infection the adenovirus E1A genes regulate expression of the other early viral genes by a *trans* mechanism via the protein product and by a *cis* mechanism directly. The E1A polypeptides are also capable of stimulating expression of a large number of cellular genes when they are transfected into target cells, although not when they are integrated into the cellular chromatin. This stimulation is non-specific to the extent that there appear to be no common DNA sequences upstream of the target genes (either viral or cellular) and similar activation has been observed with the herpes virus immediate early gene product (Imperiale *et al.*, 1983; Gaynor *et al.*, 1985). The E1A proteins could accomplish the activation process by producing increased levels of rate-limiting transcriptional factors which are required for activity of RNA polymerase III and possibly RNA polymerase II, as has been suggested by Hoeffler & Roeder (1985) and Yoshinaga *et al.*, (1986). The activities of these polymerases could result in the preferential transcription of 'foreign genes' within the cell, either infected or transfected. Unfortunately, this scheme does not explain the increased transcription of host cell genes integrated into the host chromosome (e.g. HSP70, thymidine kinase) or the preferential expression of other viral early genes integrated into the host cell chromosome.

An alternative mechanism to that seen after infection probably occurs in transformed cells since the response of cellular proteins to E1A in the two systems may be totally different (e.g. MHC class I antigen is reduced in Ad 12 transformants, but not Ad 12 infected cells). Thus it must be remembered that host cell type, the cellular gene examined and virus serotype can all be determining factors when examining the regulatory mechanism of the E1A genes.

(c) Biochemical activities associated with the E1 proteins

(i) Protein kinase activity. When it was observed that the 60 kDa polypeptide of *v-src* (pp60^{v-src}) was a protein kinase (Collett & Erikson, 1978), a number of workers investigated the E1 proteins of adenovirus for similar activity. It was found that immunoprecipitated Ad 5 and Ad 12 E1 polypeptides, when incubated with [γ -³²P]ATP, were capable of phosphorylating histone H3 and immunoglobulin heavy chain at low levels (Lassam *et al.*, 1979; Raska *et al.*, 1979; Branton *et al.*, 1981). Using specific antisera the kinase activity was found to be associated with both the E1A polypeptides (Branton *et al.*, 1985) and the larger E1B polypeptide (Yee & Branton, 1983) in Ad 5 infected and transformed cells. However, it should be noted that other workers have failed to detect any kinase activity whatsoever associated with the E1 polypeptides in Ad 12 transformed rat and human cells (Grand & Gallimore, 1984). Similarly, none of the E1 proteins appear to be able to bind ATP (Grand & Gallimore, 1984; Branton *et al.*, 1985) as might be expected for *bona fide* protein kinases.

(ii) Other activities. A very large number of mutants of the C group adenoviruses have been constructed in attempts to provide information on the functions of the early region 1 proteins. These have, however, mainly provided data relating to the general phenomenon of transformation rather than to particular biochemical activities (see section 3). Partial exceptions to this, however, are the *cyr*⁺ (cytotoxic) mutants of Ad 12 (Takemori *et al.*, 1968, 1969) and the *lp*⁺ (large plaque) mutants of Ad 2 (Chinnadurai *et al.*, 1979; Chinnadurai, 1983; Takemori *et al.*, 1984) which are characterized by extensive cytopathic effects in infected cells such that the mutant causes larger clearer plaques than does wild type virus. These mutations which result in the degradation of cellular and viral DNA have been mapped in both virus types to the smaller E1B gene (Laifatt & Mak, 1982; Mak & Mak, 1983; White *et al.*, 1984; Pilder *et al.*, 1984; Subramanian *et al.*, 1984; Takemori *et al.*, 1984; White & Stillman, 1985). Presumably the nuclease which is responsible for DNA degradation in the mutant infected cells is under the control of the E1B protein following wild type infection. In the mutants the E1B 163 aa (in Ad 12) or E1B 175 aa (in Ad 2) proteins are absent, allowing the nuclease to act unhindered. These observations leave a number of interesting questions unanswered. Firstly, whether this enzyme is of cellular or viral origin, although the latter possibility seems more likely; secondly, whether this nuclease regulatory function is compatible with the presence of the protein in the cellular membranes and thirdly whether the anti-nuclease activity is required or maintained in transformed cells.

A number of other Ad 5 mutants have been isolated with lesions in the E1B DNA which give clues to the possible functions of the E1B polypeptides (Babiss & Ginsberg, 1984; Babiss *et al.*, 1985). It has been concluded, from a study of H5 *dl* 118 (which produces no E1B protein) and H5 *dl* 163 (which fails to produce the larger E1B protein) that the E1B 496 aa polypeptide is required for accumulation of virus mRNA and for the reduction in host cell mRNA transport and translation. Similar conclusions have been drawn by Pilder *et al.* (1986) on the basis of another Ad 5 E1B mutant (*dl* 338).

Ad 12 E1B mutants also fail to replicate and produce late proteins in detectable quantities (Gallimore *et al.*, 1986a; Shiroki *et al.*, 1986) in contrast to E1A mutants in which the lytic cycle does go to completion albeit much more slowly than for wild type virus. Interestingly, the Ad 12 mutants (H12 *in* 602, H12 *del* 620, H12 *hr* 703, H12 *hr* 704), with lesions in the 482 aa E1B protein, do not make any truncated or deleted 482 aa E1B products, or the 163 aa E1B protein; they also produce only low levels of the E1A polypeptides. However, E1B mutant infections carried out on Ad 12 E1A HER 1 cells (an Ad 12 E1A-transformed human embryo retinal cell line which constitutively expresses the E1A polypeptides) are characterized by wild type levels of the 163 aa E1B protein (R. J. A. Grand & P. H. Gallimore, unpublished work). It has therefore been concluded that there exists in Ad 12 an additional regulatory system controlling the expression of viral genes such that the E1B 482 aa protein is required not only for the expression of viral late genes, but also for full expression of the E1A polypeptides which in turn control the levels of the E1B protein (see Fig. 3). There appears to be no control of E1A by the E1B 496 aa polypeptide in Ad 5 since expression of the 175 aa protein is at wild type levels in *dl* 163 (Babiss & Ginsberg, 1984).

(d) Protein-protein interactions involving the E1 proteins

It is now well established that many proteins do not exert their influence on cellular metabolism via a direct enzymic activity, but rather by means of subtle interactions with other cellular polypeptides which may either have enzymic functions of their own or be cellular regulators in their own right. Therefore, some effort has been expended recently in attempts to discover what cellular (or viral) proteins bind to the products of E1 either in infected or transformed cells. An initial approach to this problem has been to immunoprecipitate the E1 polypeptides with specific antisera and to see what other proteins co-precipitate. When this technique was applied to Ad 5 transformed mouse cells (Sarnow *et al.*, 1982b) it was found that there was a physical association between the Ad 5 E1B 496 aa protein and cellular p53 tumour antigen (see Crawford, 1983, for review) which is known to bind to SV40 large T. Levels of p53 in normal cells have been shown to vary throughout the cell cycle, reaching a maximum in late G1 (Milner & Milner, 1981; Reich & Levine, 1984). In many transformed lines, however, levels of the protein are much higher, probably due to increased stability which has been attributed, in the case of SV40 transformants, to binding to SV40 large T (Reich & Levine, 1984). Although the Ad 5 E1B 496 aa polypeptide binds strongly to p53, the comparable Ad 12 protein is bound either not at all or very weakly in transformed rat cells (Zantema *et al.*, 1985a). The p53 polypeptide is detected at high level (presumably due to an increased stability) in both Ad 5 and Ad 12 transformed rodent cells expressing the larger E1B protein, but not in those expressing only E1A (Zantema *et al.*, 1985a). However, Zantema *et al.* (1985a) found that those cell lines transformed with DNA encoding the E1A+E1B 175 aa protein expressed levels of p53 intermediate between the E1A transformant (or normal cells) and the E1 transformants. This observation is rather difficult to reconcile with the idea that p53 levels are high as a result of the expression of the E1B large 496 aa polypeptide. Whether this indicates that p53 production is a consequence of transformation rather than the presence of the larger E1B polypeptide is not clear at present. In those transformants where the E1B 496 aa protein-p53 complex exists (i.e. the Ad 5 lines) both components are detected in 'cytoplasmic bodies' (Zantema *et al.*, 1985a, b), whereas where no complex can be detected (Ad 12) both proteins are in the nucleus (Grand & Gallimore, 1984; Zantema *et al.*, 1985b).

The situation in adeno-transformed human cell lines is rather different. In those cells in which the Ad 12 E1B 485 aa protein is expressed, p53 can be detected in the nucleus (Grand & Gallimore, 1984) at levels comparable with those found in SV40-transformed HERs (Grand & Gallimore, 1986). However, in the Ad 12 transformed cells where the E1B 485 aa polypeptide is absent, normal cellular amounts of p53 are observed. In all the Ad 2/Ad 5 transformants examined only low levels of p53 could be detected irrespective of E1B expression (Grand & Gallimore, 1986). It is also important to note that there is no increase in p53 as a consequence of the presence of E1B proteins during viral infection of human cells (Sarnow *et al.*, 1982b; Grand & Gallimore, 1986); therefore it must be concluded that prolonged rather than transient expression is essential before p53 concentration is increased.

A second molecule which has been shown to co-immunoprecipitate with the Ad 2/Ad 5 495 aa E1B protein is the E4 25 kDa polypeptide (Sarnow *et al.*, 1982a, 1984). It appears possible that these two proteins may form a functional complex which is involved in the accumulation of viral late messages and the shut-off of host cell protein synthesis during infection. As outlined above (section 5c) those adenovirus mutants which do not express the larger E1B protein have deficiencies in viral mRNA accumulation and fail to reduce host-cell mRNA production (Babiss & Ginsberg, 1984; Babiss *et al.*, 1985; Pilder *et al.*, 1986). Similar phenotypes have been observed for Ad 5 E4 (Halbert *et al.*, 1985) and Ad 2 E4 mutants (Weinberg & Ketner, 1986).

It seems clear therefore that the regulatory function of E1B on the levels of mRNAs is well established during infection, and that this is achieved by means of an E4-E1B 495 aa protein complex. It should be noted, however, that the E1B 495 aa protein binds only to the E4 25 kDa polypeptide and not to any of the other proteins which have been predicted from E4; for example, the 11 kDa polypeptide characterized by Sarnow *et al.* (1982c).

Using Western blotting procedures it has been shown that the Ad 12 E1B 485 aa protein also interacts with itself to form dimers and, under some circumstances, polymers (Grand & Gallimore, 1984). This interaction is stabilized through intermolecular disulphide links and probably leads to the formation of a 100 kDa molecule *in vivo*.

Co-immunoprecipitation studies have also been carried out using E1A-specific antisera, but with, as yet, less clear-cut results than those described above for E1B. Yee & Branton (1985b) have shown that five major protein species (of 250 kDa, 105 kDa doublet, 68 kDa and 65 kDa) can be immunoprecipitated from Ad 5 infected and transformed cells together with the E1A gene products. These polypeptides were all of cellular origin. The two smallest proteins appeared to bind most strongly to the 289 aa E1A polypeptide whereas the remainder bound to both major E1A proteins equally. Little is known about any of these E1A binding proteins except that the 250 kDa and 105 kDa species contain covalently bound phosphate groups.

An alternative approach to the recognition of adenovirus E1 binding proteins has been to select possible cellular targets and then to examine whether the potential interaction actually takes place *in vitro* (Grand & Gallimore, 1984). A number of cellular proteins were examined and it was found that the partially purified Ad 12 E1A polypeptides bound strongly to a number of histones, with H1 and H4 forming a tight complex which persisted even under denaturing conditions. Whilst it is tempting to speculate that E1A could exert an influence on transcription through a histone interaction there has been no evidence to suggest that such a complex exists *in vivo*. However, this observation probably explains the finding by Fergusson *et al.* (1985b) that the purified Ad 2/Ad 5 E1A 289 aa protein does not bind to DNA alone, but does bind in the presence of a HeLa cell nuclear extract. It is reasonable to suppose that such an interaction could be mediated through histones.

There are a number of pitfalls associated with all these sorts of investigations. The major one is that complexes between the E1 proteins and the identified target protein may have been created by the processing used for

detection. For example, in the immunoprecipitation studies it is possible that disruption of the cell leads to juxtaposition of proteins which are normally spatially separated in different organelles. A second problem may be that conditions stringent enough to reduce background non-specific interactions may also cause the break-up of the adenovirus E1-host cell protein complex; there is little evidence to suggest that physiologically important interactions are necessarily resistant to high ionic strength or denaturing conditions. These and a number of other factors must be taken into account when assessing the validity and worth of experiments designed to demonstrate E1 binding proteins.

6. CONCLUSIONS

Over the past decade it has been shown that the expression of the four major adenovirus early region 1 proteins is sufficient to produce a transformed phenotype in a large number of target cell types. How these proteins act to change cellular metabolism, morphology and growth characteristics, however, is largely unknown. Our knowledge of E1 polypeptide function is based almost exclusively on studies of lytic infection where they almost certainly have additional and/or alternative functions. Of course, there will be features of their activity in the two situations in common, but it is probable that precise modes of action will be different. For example, the 'trans' regulatory actions of the E1A polypeptides is likely to be as important in transformation as in infection, but how and why only a limited number of cellular genes are selectively activated remains unclear. Conversely the E1B proteins during lytic infection have the ability to control DNA degradation, shut down host cell protein synthesis and selectively to process or stabilize viral mRNA, but as yet there has been no suggestion that such roles feature in the acquisition of the transformed phenotype.

The characterization of the mode of action of E1 proteins during infection and transformation is still the most difficult puzzle facing adenovirus molecular biologists. Possible ways of tackling the problem in the future are many, but it is likely that two recently developed techniques will be most productive, in the short term at least. The first of these is the use of expression vectors to make large quantities of viral protein which can easily be purified and then used in conventional biochemical studies. Initial investigations by Fergusson *et al.* (1985a,b) and Lucher *et al.* (1985) using this technique, coupled with an activity assay which has been developed, have yielded very encouraging results. The second method is to create specific mutants, using site-directed mutagenesis, with lesions in areas of the proteins which the biochemical studies have suggested may be of particular importance (for example the work of Montell *et al.*, 1982, 1984). Hopefully these studies will lead to a clarification of the biochemical activities of the individual adenovirus proteins which distinguish serotypes on the basis of tumorigenicity. The elucidation of adenovirus transformation may, in turn, help us to achieve a wider understanding of oncogenesis in general.

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