The EMBO Journal vol.7 no.3 pp.825-833, 1988

Analysis of HPV-1 E4 gene expression using epitope-defined antibodies

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Communicated by L.V.Crawford

Six monoclonal antibodies (mAbs) have been raised against the E4 proteins of HPV-1. Five of these were found to recognize denaturation-resistant epitopes as determined by Western blotting—and their binding sites were identified by determining their reactivity against a panel of bacterial E4−β-galactosidase fusion proteins which contained progressive deletions at the C-terminal end of the E4 region. The five mAbs were found to bind to four distinct sites. By using these epitope-defined mAbs, along with anti-peptide antibodies raised against putative N- and C-terminal E4 sequences, we have determined the relationships between the eight distinct polypeptides (mol. wt 10/11 kd, 16/17 kd, 21/23 kd and 32/34 kd) previously shown to be expressed from the E4 gene of HPV-1 in productively infected papillomas. The 17 kd E4 polypeptide appears to be the product of a spliced mRNA encoding five amino acids from open reading frame (ORF) E1 joined onto 120 from the E4 ORF. The 16 kd and 10/11 kd proteins, which may be derived from this, lack sequences (~15 and 70 amino acids respectively) encoded by the 5′ end of the E4 gene. The 32/34 kd proteins were detected by all antibodies which reacted with the 16/17 kd polypeptides, suggesting that they represent dimers of the latter species. The 21/23 kd polypeptides, however, do not appear to be simple dimers of the 10/11 kd protein as previously predicted, and reacted with antibodies whose epitopes mapped in the N-terminal half of the E4 protein. A major epitopic site (PEVPEVEDE) has been located in the 10/ 11 kd protein. Using an antibody specific for the 17 kd (and 34 kd dimer) protein, the 17 kd polypeptide was only detected in the nucleated layers of wart tissue and was absent from the stratum corneum. mAbs which recognized the 16 and 17 kd protein (as well as minor 21/23 and 32/34 kd species) stained all layers including the stratum corneum.

Key words: HPV-1 E4/monoclonal antibodies/papillomavirus

Introduction

Papillomaviruses are a diverse group of DNA tumour viruses which cause epithelial proliferations in man and animals (for review see Broker and Botchan, 1986; Pfister, 1984; Pfister *et al.*, 1986). Although the genomes of several human papillomavirus (HPV) types have been sequenced (Danos *et al.*, 1982; Schwarz *et al.*, 1983; Seedorf *et al.*, 1985; Dartmann *et al.*, 1986; Cole and Streeck, 1986; Fuchs *et al.*, 1986) the study of the HPVs has been restricted by the lack of a tissue culture system in which they can be successfully propagated. As a result, our knowledge of papillomavirus proteins has been derived either from the study of bovine papilloma virus type 1 (BPV-1), which has the ability to transform certain established lines of mouse fibroblasts (Lowy *et al.*, 1980) or from the analysis of viral proteins in naturally occurring tumours. This has allowed transformation, replication and regulatory functions to be assigned to several papillomavirus early open reading frames (ORFs) (Spalholz *et al.*, 1985; DiMaio *et al.*, 1986; Androphy *et al.*, 1985; Lusky and Botchan, 1985) and has enabled a number of viral proteins to be identified (see Broker and Botchan, 1985). In papillomas induced by HPV-1, however, only three viral gene products have been detected—L1, L2 and E4 (Doorbar and Gallimore, 1987; Komly *et al.*, 1986; Doorbar *et al.*, 1986). While the L1 and L2 ORFs have been shown to encode the major and minor virion structural proteins expressed in cells of the upper spinous and granular layers (Doorbar and Gallimore, 1987; Komly *et al.*, 1986), the functions of the E4 gene products have not yet been determined (Neary *et al.*, 1987). In warts induced in HPV-1, the E4 encoded proteins are particularly amenable to study, and are expressed at high levels in cells in which viral DNA synthesis is occurring (Breitburd *et al.*, 1987). In such warts, they can represent up to 30% of SDS extractable protein (Doorbar *et al.*, 1986) and are associated with the abundant cytoplasmic and nuclear inclusion granular pathognomic of HPV-1-induced papillomas (Doorbar *et al.*, 1986; Breitburd *et al.*, 1987). Although originally classified as early gene products, the abundance and distribution of the E4 polypeptides in naturally occurring tumours suggests that they do not in fact represent typical tumour virus early proteins (Doorbar *et al.*, 1986; Breitburd *et al.*, 1987). At least eight distinct protein species have been identified as being derived from the HPV-1 E4 ORF. The major E4 gene products comprise a protein doublet of mol. wt 16/17 kd although minor E4 species of 10/11 kd, 21/23 kd and 32/34 kd have also been identified and are variably present in HPV-1 induced papillomas (Doorbar *et al.*, 1986). In order to determine the relationship between these gene products we have prepared monoclonal and anti-peptide antibodies against the naturally occurring HPV-1 E4 proteins. By using a panel of bacterially expressed HPV-1 E4 gene fragments, the binding sites of individual monoclonal antibodies (mAbs) has been determined. This has allowed us to investigate the relationship between certain of the E4 gene products and to investigate their expression in naturally occurring tumours.

Results

Production of monoclonal antibodies to HPV-1 E4 gene products

Thirty out of 960 HAT-resistant hybridoma cell colonies

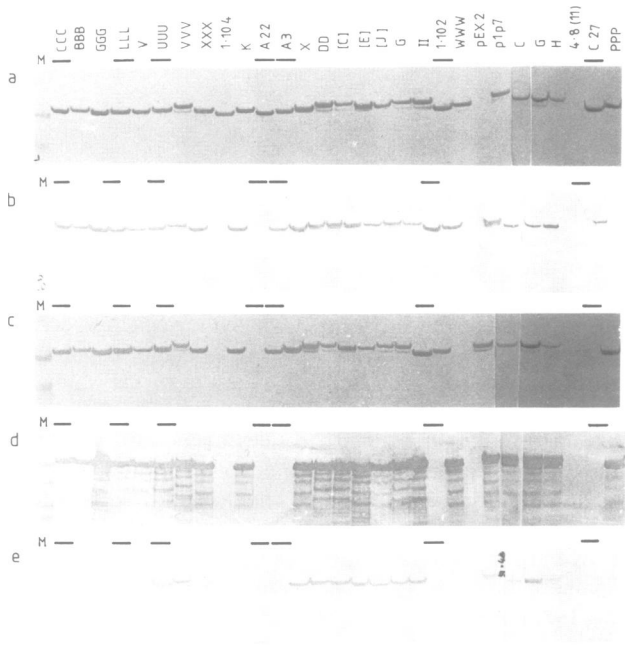

Fig. 1. Reactivity of mAbs against E4 fusion protein deletions. (**a**) Western blot using mAb 7.76 to a panel of E4 fusion proteins after SDS gel electrophoresis. Mol. wt markers (116 kd, 84 kd, 58 kd, 48.5 kd) are indicated in track M. Positive and negative controls are present in tracks p1p7 and pEX2 respectively. Identical blots are shown in (**b**), (**c**), (**d**) and (**e**) except that blotting was carried out using MAbs 9.60, 9.95, 4.37 and 8.40 respectively. Bars (—) indicate tracks containing fusion proteins important in defining epitopic sites.

were found to be reactive against the purified 16/17 kd HPV-1 E4 protein when tested in a preliminary ELISA screen. Five of these (4.37, 7.76, 8.40, 9.60 and 9.95) recognized denaturation resistant epitopes, as determined by Western blotting to extracts of HPV-1 induced warts, and were subsequently recloned and further characterized. All five mAbs were found to have activity in Western blots against a fragment of the HPV-1 E4 protein (102/126 amino acids encoded by the centre of the E4 ORF) when expressed as fusion proteins linked to β-galactosidase (expressed from clone p1p7, see Doorbar *et al.*, 1986). The ability of these mAbs to react with denatured protein (from warts) and also with denatured fusion proteins strongly suggests that they recognize sequential epitopes which are not dependent on protein conformation. Hybridoma supernatant from clone 4.37 (IgG2a) was found to work efficiently in Western blots at a dilution of 1/200, while supernatant from clones 9.95, 9.60 (IgG1), 7.76 (IgM) and 8.40 (Ig type not determined) had optimal activity at a dilution of 1/20. Supernatant from hybridomas which were not positive in the initial ELISA assay were pooled into groups of 10, and screened against HPV-1 papilloma extracts by Western blotting in order to detect possible activity against minor E4 encoded species. No antibodies were detected, however, which reacted solely with the 10/11 kd, 21/23 kd or 32/34 kd E4 proteins.

Mapping the epitopes of anti-E4 monoclonal antibodies

In order to identify the epitopes recognized by the five anti-E4 mAbs, a series of bacterial expression constructs were prepared which contained the HPV-1 E4 gene, but with progressive deletions at its 3′ end. Sixty-four individual expression clones were identified, capable of expressing E4 protein fragments from between 15 to 102 amino acids in length. A disproportionately large number of recombinants (10/64) were found to terminate at the same position in the E4 sequence (around nucleotide 160 of the E4 ORF) suggesting that this region may represent an area of instability for exonuclease *Bal*31.

The reactivity of each mAb was determined by Western blotting against the panel of SDS denature fusion protein deletions (Figure 1), and the results are summarized in Figure 2a. By identifying the largest E4 fusion protein with which each mAb reacted, it was possible to identify the linear epitope recognized by each antibody. The five monoclonals were found to bind to four distinct sites along the E4 protein (Figure 2a).

To determine the antibody binding sites more precisely, the 3′ ends of the E4 inserts from clone UUU (8.40 +ve), LLL (8.40 −ve), GGG (4.37 +ve), 1.102 (4.37 −ve), A3 (9.95/9.60 +ve), A22 (9.95/9.60 −ve) and C27 (7.76 +ve) were confirmed by DNA sequence analysis.

No difference was observed between the reactivity of mAbs 9.95 and 9.60; both these antibodies reacted with fusion protein A3 but not with A22. These fusion proteins differ in their E4 content by only two amino acids, strongly suggesting that these two mAbs recognize the same epitope.

Differential reactivity of mAbs against naturally occurring E4 gene products

The reactivity of the five mAbs was determined by Western blotting to extracts of HPV-1-induced papillomas and by immunofluorescence. Antibody 8.40 (recognition site at amino acid residue ~78, see Figure 7) reacted with all the HPV-1 E4 gene products (10/11 kd, 16/17 kd, 21/23 kd, 32/34 kd) and showed a pattern of staining identical to that observed with a polyclonal antibody prepared against purified 16/17 kd (E4) protein (Figure 3). mAbs whose epitopes lay in the N-terminal half of the E4 protein (amino acid residue 1→65) were found not to recognize the smaller 10/11 kd E4 gene products, and had reactivity against only the 16/17 kd, 21/23 kd and 32/34 kd proteins (Figure 3). This suggests that the 10/11 kd proteins contain E4 sequences derived only from within the C-terminal half (amino acid residues 65→126) of the E4 ORF. It is interesting to note that a possible splice acceptor site, 'TTACGGACGGCG-AAG/A' (nucleotide 3398−3414 in HPV-1 sequence, nucleotide 0 at *Hpa*I site, Danos *et al.*, 1982; Mount, 1982) exists between the 4.37 and 8.40 mAb epitope coding regions which could give rise to the 10/11 kd gene products (see Figure 7).

Reactivity of E4 polyclonal antibodies against E4 fusion protein deletions

To confirm that the 10/11 kd E4 polypeptides (but not the 16/17 kd species) lack sequences derived from the 5′ half of the E4 ORF, polyclonal antibodies were prepared against the 10/11 kd and 16/17 kd proteins after purification from a HPV-1-induced wart, as described previously (Doorbar *et al.*, 1986). Polyclonal antibodies raised against the 16/17 kd E4 species were found to react with all the E4 fusion proteins although, as expected, reactivity was less against proteins containing smaller fragments of E4 (Figure 4). Antiserum raised against the 10/11 kd doublet reacted only with those fusion proteins which contained C-terminal E4

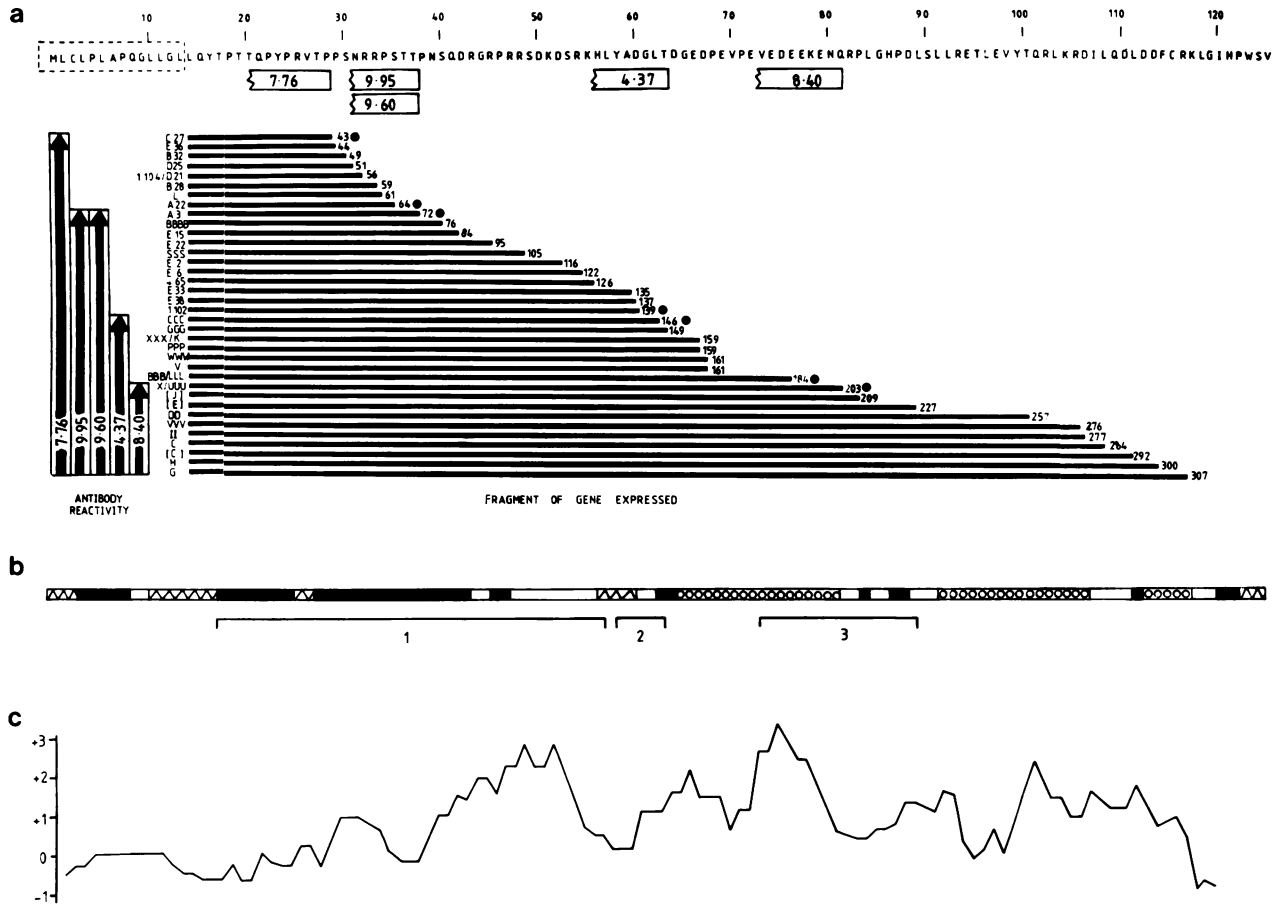

Fig. 2. (**a**) Determination of mAb binding sites. The complete amino acid sequence encoded by the unspliced HPV-1 E4 ORF (as predicted by Danos *et al.*, 1982) is illustrated at the top of the figure. The black bars beneath indicate portions of the E4 protein expressed at high level in *E.coli*. The designation of each deletion construct is indicated at the left of each bar (C27 to G), and insert size in base pairs (from *PstI* site at 3227 bp in HPV-1 sequence), at the right (43 to 307). The first 14 E4 amino acids, which were not expressed from any of the constructs, are enclosed in a dashed box. Vertical arrows at the left of the figure illustrate the extent of reactivity of the five mAbs, with the panel of E4 fusion proteins. The approximate positions of mAb epitopes deduced from this are represented as boxes beneath the E4 amino acid sequence. Inserts whose 3′ ends were confirmed by DNA sequence analysis are indicated by black circles. (**b**) Secondary structure of HPV-1 E4 protein and epitope prediction analysis based on Protean I program (see Materials and methods). ■ = region of random coil, ▨ = region of β-pleated sheet, ▩ = region of α helix and □ = region in reverse turn configuration. Predicted epitopes, 1st, 2nd and 3rd (in order of likely importance), are indicated beneath the secondary structure. (**c**) Hydrophilicity analysis of HPV-1 E4 protein according to Hopp and Woods (1981). Likely epitopes are represented by hydrophilic peaks. The highest peak corresponds to a major epitope in the 10/11 kd protein.

fragments (Figure 4). A sharp cut-off was observed between fusion proteins which reacted with anti-10/11 kd antiserum (LLL/BBB and larger) and those which did not (WWW and smaller), suggesting that the 10/11 kd protein contains an immunodominant site situated between amino acids 68 and 76 (see Figure 4, amino acid residue numbers are indicated in Figure 2). This observation is consistent with epitope prediction analysis which indicates that the predominant E4 antigenic sites are located between amino acid residues 18 to 88 (Figure 2b), and hydrophilicity studies which predict a major antigenic site at residue ~73 (Figure 2c).

Analysis of E4 gene expression using anti-synthetic peptide antibodies

In order to define the N and C termini of the 16 and 17 kd polypeptides, a series of synthetic peptides were prepared corresponding to the protein sequences encoded by the 5′ and 3′ ends of the E4 ORF. Recent studies have identified an mRNA species in naturally occurring HPV-11-induced papillomas, which has the ability to splice five amino acids derived from the E1 ORF onto a large region encoded by

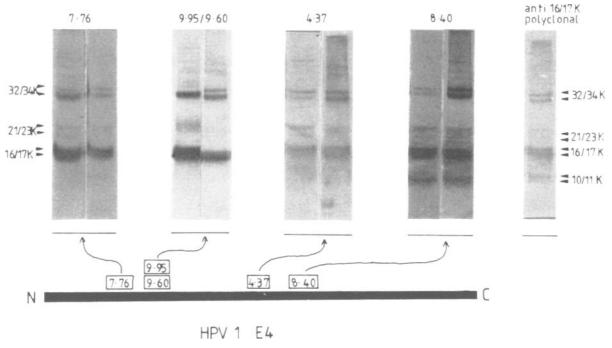

Fig. 3. Reactivity of site-defined mAbs against naturally occurring HPV-1 E4 gene products. Tracks represent Western blots after SDS gel electrophoresis of extracts of HPV-1-induced papillomas. From left to rights, blots are stained with mAb 7.76, 9.95/9.60, 4.37, 8.40 and anti-16/17 kd polyclonal antiserum. The positions of the 32/34 kd, 21/23 kd and 16/17 kd E4 proteins (detected by all antibodies) are indicated at the left and right. The position of the 10/11 kd protein, visible only after staining with mAb 8.40 and anti-16/17 kd polyclonal antiserum, is indicated on the right. The approximate locations of MAb binding sites on the E4 protein are illustrated at the bottom of the figure.

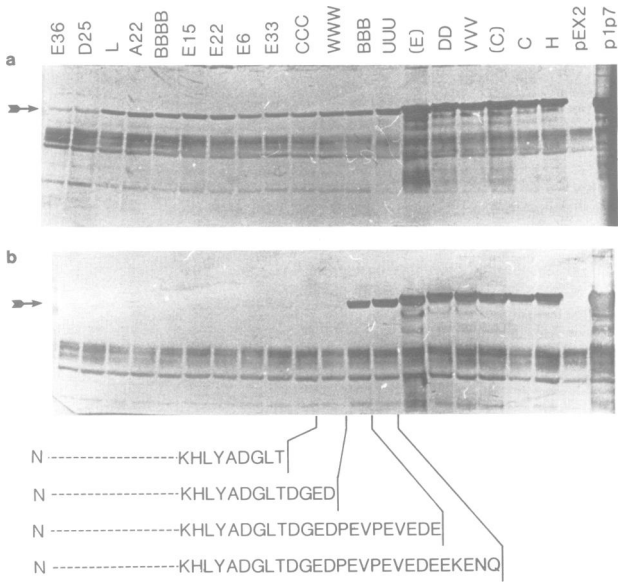

Fig. 4. Reactivity of anti-16/17 kd and anti-10/11 kd polyclonal antibodies against bacterially expressed E4 protein fragments. (**a**) SDS gel electrophoresis and Western blotting using anti-16/17 kd antiserum of crude bacterial extracts containing E4 fusion proteins of various sizes (arrowed). The fusion protein containing the smallest E4 N-terminal insert is located on the left of the blot (E36). Proteins containing inserts of increasing size are present in tracks to the right of this (see Figure 2) (D25−H). Positive (p1p7) and negative (pEX2) controls are illustrated in the rightmost two tracks. (**b**) Identical blot to that shown in (a) except that staining was carried out using anti-10/11 kd antiserum. The positions of the fusion proteins are arrowed. The protein sequence encoded by the 3′ ends of the E4 inserts present in clones CCC and WWW (anti-10/11 kd −ve) and BBB and UUU (anti-10/11 kd +ve) are shown at the bottom of the figure. The dotted line indicates protein sequences common to all four fusion proteins.

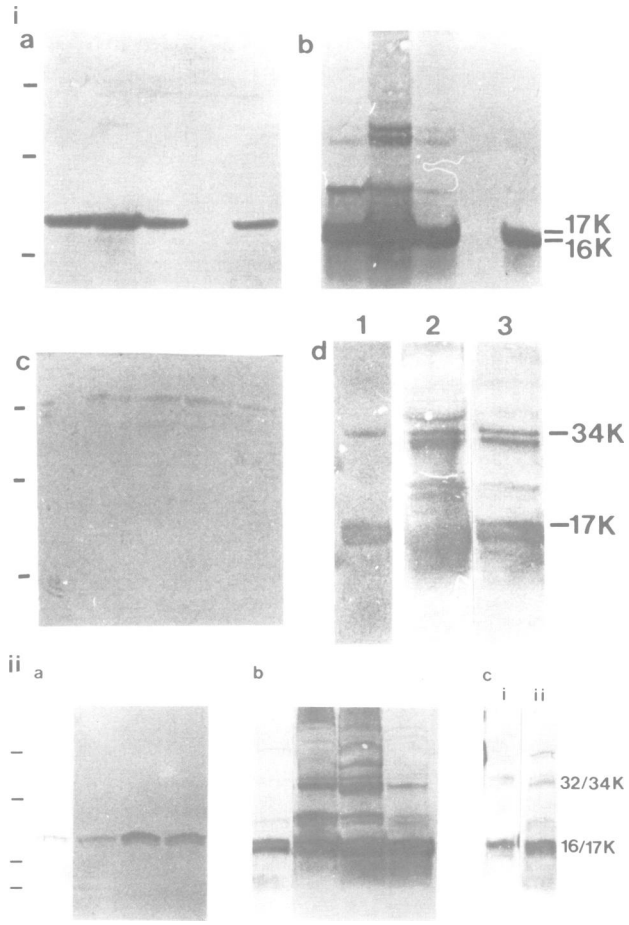

Fig. 5. (**i**) Reactivity of anti-E4 (spliced N terminus) and anti-E4 (unspliced N terminus) synthetic peptide antisera against naturally occurring E4 gene products. (**a**) Western blot of extracts of HPV-1-induced warts following SDS gel electrophoresis and staining with anti-E4 (spliced N terminus) antiserum. The second track from the right contains an extract of a HPV-2-induced wart. Specific detection of the 17 kd E4 protein was observed in all HPV-1 extracts. Mol. wt markers (68 kd, 25.7 kd, 12.3 kd) are indicated on the left. (**b**) Identical blot to that shown in (a) except that staining was carried out using mAb 4.37. The 16/17 kd proteins are indicated. (**c**) Identical blot to that shown in (a) except that blotting was carried out using anti-E4 (unspliced N terminus) antiserum. No specific staining was observed. (**d**) Specific detection of 34 kd E4 protein (as well as 17 kd) by Western blotting using anti-E4 (spliced N terminus) antiserum (**track 1**). **Tracks 2** and **3** contain different loadings (25 μg and 10 μg) of the same extract after blotting to polyclonal anti-16/17 kd antiserum. (**ii**) Reactivity of anti-E4 (C terminus) antiserum against naturally occurring E4 gene products. (**a**) Western blot of extracts of HPV-1-induced warts following SDS gel electrophoresis, and staining with anti-E4 (C terminus) antiserum. The 17 kd E4 protein is readily detectable in all tracks—the 16 kd polypeptide is variably detected. The positions of mol. wt markers (68 kd, 25.7 kd, 12.3 kd, 6.2 kd) are indicated at the left. (**b**) Identical blot to that shown in (a) except that staining was carried out using anti-16/17 kd polyclonal antiserum. (**c**) Detection of 32/34 kd as well as 16/17 kd protein by Western blotting using anti-E4 (C terminus) antiserum (**track i**). **Track ii** represents an identical blot after staining with anti-16/17 kd polyclonal antiserum. The positions of the 32/34 kd and 16/17 kd E4 protein are indicated on the right.

ORF E4 (Chow *et al.*, 1987a). To investigate if any of the HPV-1 E4 gene products contained such N-terminal E1 sequences, two synthetic peptides were prepared, corresponding to the N-terminal regions of either the putative unspliced E4 protein (peptide LCLPLAPQGLL), or to the product of a spliced E1/E4 mRNA (peptide ADNKAPQGLLC, see Figure 7). The peptides were linked to keyhole limpet haemocyanin, bovine thyroglobulin, bovine serum albumin and ovalbumin using three different methods of linkage—via disulphide bonding at the cysteine residue, linkage via amino groups using glutaraldehyde, or C-terminal linkage using ECDI (see Materials and methods). In each case high titre antisera were obtained against both peptides as determined by an ELISA assay. In Western blots, antibodies prepared against the E1/E4 'spliced' N-terminal peptide were found to react specifically with only the 17 kd and 34 kd E4 gene products, and showed no reactivity against other HPV-1 E4 species [Figure 5(i)a]. Antibodies prepared against the unspliced N-terminal peptide showed no anti-E4 reactivity by Western blotting [Figure 5(i)c]. Identical reactivities were obtained irrespective of which carrier protein or method of linkage was used. This suggests that the 17 kd (and 34 kd) E4 protein is the primary product of a spliced E1/E4 mRNA, and that the 16 kd E4 protein lacks N-terminal sequences derived from either the 5′ end of the E4 ORF or from an E1/E4 spliced mRNA. As the 17 kd protein contains a blocked N terminus (Doorbar *et al.*, 1986) it has not been possible to confirm the N-terminal sequences directly by protein sequencing.

The possibility exists that the 16 kd protein arises from the 17 kd product by N-terminal cleavage. Clusters of leucine residues (LLXLL) which are a conserved feature at the N terminus of the HPV E4 proteins are frequently present

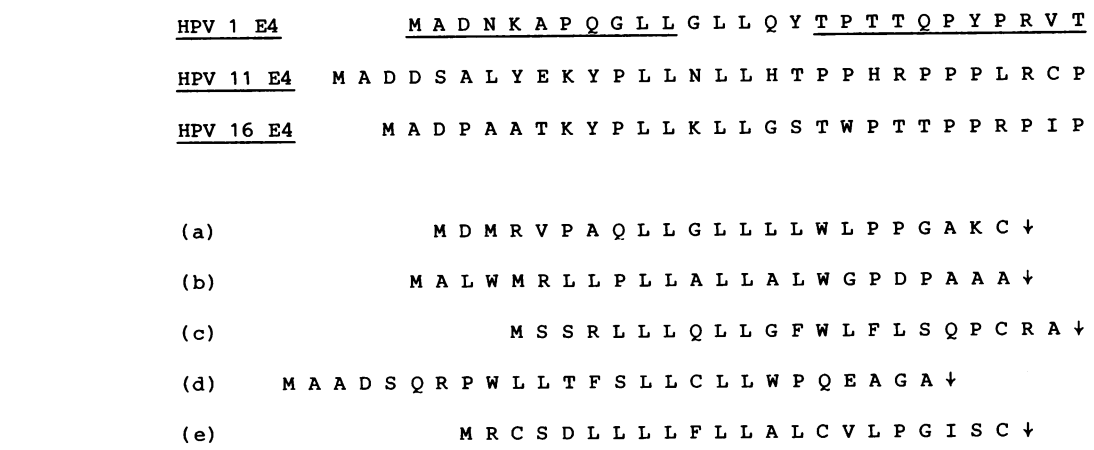

Fig. 6. Alignment of leucine clusters (LLXLL) at the N terminus of HPV E4 proteins. The N-terminal sequences of the HPV-1, -11 and -16 E1/E4 spliced gene products (as predicted by Chow *et al.*, 1987a,b) are illustrated at the top of the figure. Regions underlined in the HPV-1 sequence correspond to approximate binding sites of antibodies raised against the E1/E4 'spliced' peptide (17 and 34 kd reactive) and mAb 7.76 (32/34 kd, 21/23 kd and 16/17 kd reactive). Sequences a, b, c, d and e represent the N-terminal sequences of proteins which are known to be cleaved at their N terminus (↓ = cleavage site). Alignment is via leucine clusters. The N-terminal sequences are (**a**) Ig kappa precursor (human), (**b**) pro-insulin precursor (human), (**c**) prorelaxin precursor (rat), (**d**) somatotropin precursor (rat), (**e**) acetylcholine receptor precursor (chicken).

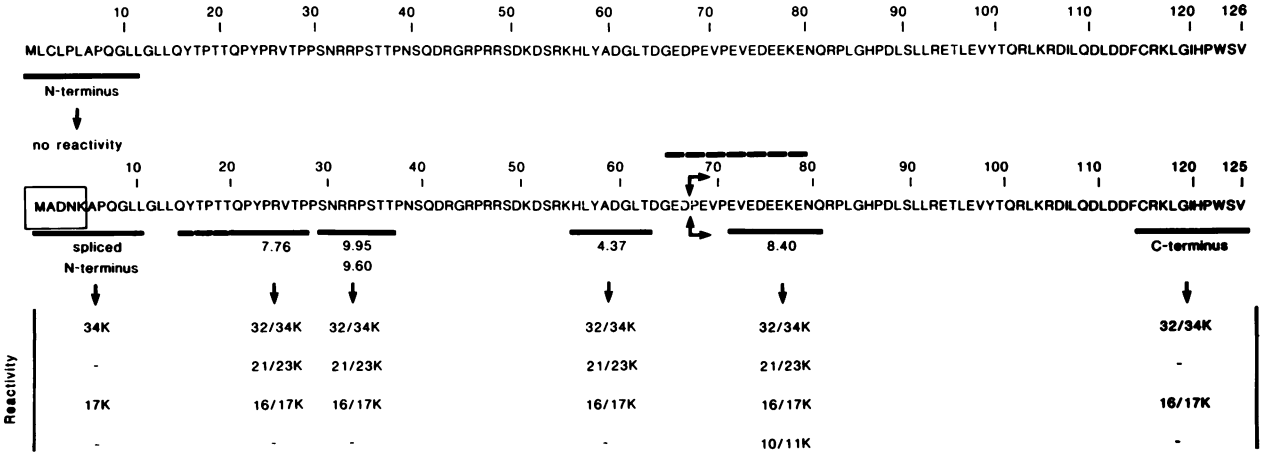

Fig. 7. Location of antibody binding sites, and anti-E4 reactivity. The amino acid sequence encoded by the unspliced HPV-1 E4 ORF is indicated at the top of the figure, and the sequence encoded by a spliced E1/E4 mRNA (Chow *et al.*, 1987a,b) shown beneath. Amino acid residues derived from the E1 ORF are boxed. The location of epitopes recognized by mAbs, and the positions of peptides used to prepare anti-peptide antibodies, are indicated by unbroken bars. The reactivity of antibodies directed against these sites is summarized beneath the vertical arrows. The glutamic acid rich sequence which may represent a proteolytic cleavage site involved in generation of the 10/11 kd proteins is indicated by a dashed line. The double arrow (↓→) defines the extent of the C-terminal E4 fragment contained in the 10/11 kd protein, if it arises from a spliced mRNA as discussed in the text.

in proteins whose N termini are known to be cleaved (Figure 6). In the latter instances the leucine residues comprise part of the signal sequence involved in protein secretion (Heijne, 1985).

The 34 kd polypeptide has previously been suggested to be a dimer of the 17 kd species (Doorbar *et al.*, 1986) and this is supported by the results presented above. We have been unable, however, to depolymerize the 34 kd protein by treatment with β-mercaptoethanol and alkylation as previously described (Crawford and O'Farrell, 1979) suggesting that bonding other than by cysteine residues may be involved.

A further synthetic peptide was prepared corresponding to 12 amino acids predicted to be encoded by the 3′ end of the E4 ORF (CRKLGIHPWSV). After linkage to carrier protein as described above, high titre antiserum was obtained as determined by ELISA assay. Anti-C terminus antibodies recognized both the 16 and 17 kd proteins, although reactivity was consistently stronger against the 17 kd species [Figure 5(ii)a]. Although anti-C terminus antibodies reacted poorly by Western blotting, the 10/11 kd and 21/23 kd products could not be visualized, even when the minor 32/34 kd proteins were detected [Figure 5(ii)c].

Distribution of E4 gene products in HPV-1-induced papillomas

By immunofluorescence analysis carried out on HPV-1-induced warts, all the mAbs (4.37, 9.96, 9.60, 7.76) except 8.40 produced a pattern of staining indistinguishable from that observed using a polyclonal antibody raised against the purified 16/17 kd proteins (Figure 8). Only weak staining was observed using mAb 8.40 (Figure 8) suggesting that the 8.40 epitope (see Figure 7) is partially hidden in the undenatured E4 polypeptides, a somewhat surprising finding as the 8.40 epitope corresponds to a hydrophilic peak in the

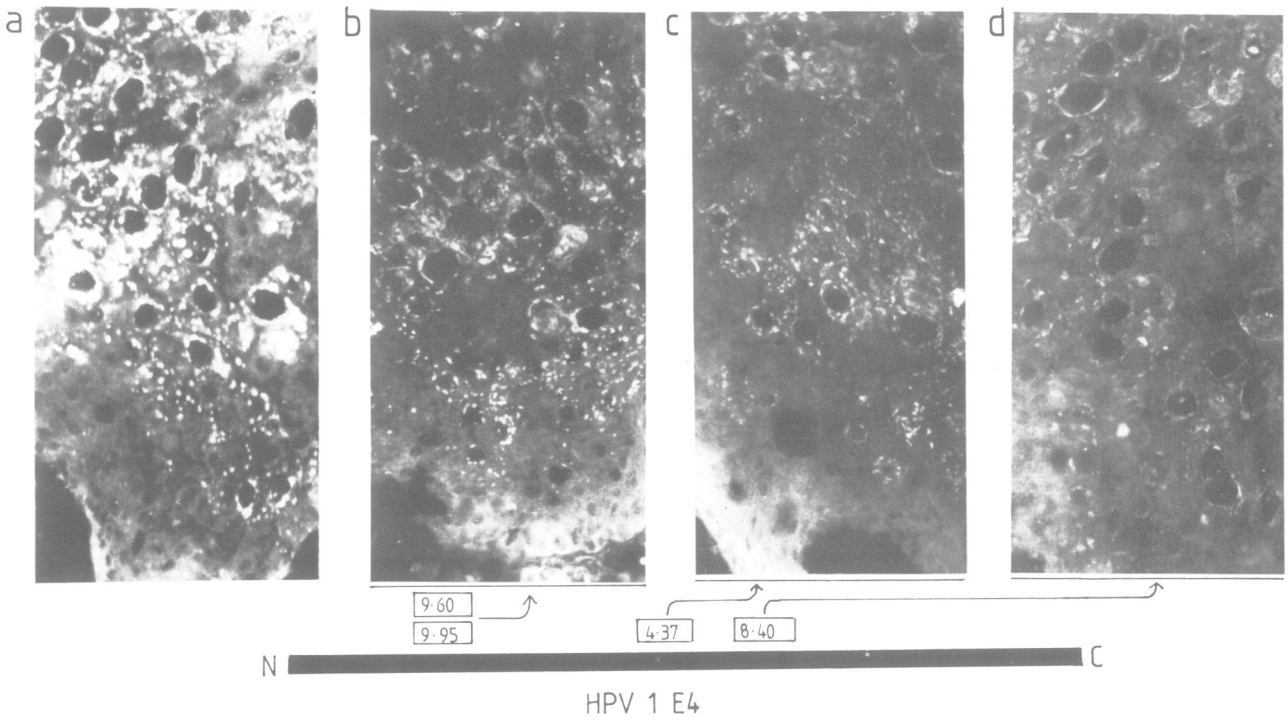

Fig. 8. Detection of E4 protein in papilloma biopsies using mAbs. (**a**)–(**d**) Adjacent sections through the lower (basal and parabasal) layers of a HPV-1-induced papilloma, after staining with (**a**) anti-16/17 kd polyclonal antiserum, (**b**) mAb 9.95, (**c**) mAb 4.37, (**d**) mAb 8.40. Cytoplasmic inclusion granules are detected by all antiserum in cells of the parabasal layer and above. Magnification ×200.

E4 molecule (Figure 2c). Conversely, mAb 7.67 which showed no reactivity by Western blotting, produced typical E4 staining on tissue sections. The E4 gene products were first detected associated with cytoplasmic inclusion granules in sporadic cells of the parabasal layer (Figure 8; see also Breitburd *et al.*, 1987), but became more widespread in cells of the spinous and granular layers. Although nonspecific background staining is often high in cells of the cornified layer, all the anti-E4 mAbs tested (4.37, 9.95/9.60, 7.76 and 7.67) were found to detect E4 proteins in this region, but produced no staining in sections of normal skin or against papillomas induced by HPV-2.

The anti-E4 (sliced N terminus) antibody, which recognized only the 17 and 34 kd E4 gene products (by Western blotting) did not produce the full E4 staining pattern, and reacted only with cells of the parabasal, spinous and lower granular layers [Figure 9(i)]. A marked 'cut-off' was apparent with no 17 kd (or 34 kd) polypeptide being detectable in cells of the upper granular layer, or the stratum corneum. This is in agreement with previous studies indicating that the 17 kd protein is depleted in more terminally differentiated cells (Breitburd *et al.*, 1987). Anti-E4 (spliced N terminus) antibodies produced typical E4 staining in parabasal layers—identical to that observed with anti-16/17 kd antibodies [Figure 9(ii)]. As expected, anti-E4 (C terminus) peptide antiserum gave rise to a staining pattern intermediate between that of the anti-E1/E4 (spliced N terminus) and anti-16/17 kd polyclonal antibodies, consistent with its reactivity in Western blots.

Discussion

We have analysed the expression of the HPV-1 E4 gene products using antibodies reactive against defined epitopes. Five mAbs have been raised against the E4 gene products of HPV-1, and their binding sites determined by analysing reactivity against denatured fusion proteins containing defined fragments derived from the HPV-1 E4 ORF. This epitope mapping technique should represent an addition to established methods of locating antigenicity, such as determination of reactivity against proteolytic cleavage products (Berzofsky *et al.*, 1980; Crumpton and Wilkinson, 1965), or by comparing reactivity against closely related proteins (Smith-Gill *et al.*, 1982). For sequential epitopes such as those examined here, considerable detail can be obtained. mAb 9.95, for instance, which reacted with a fusion protein containing 25 amino acids derived from ORF E4 (A3), did not react with a protein containing two amino acids less (A22, Figure 2a). From this it appears that the two threonine residues lost from fusion protein A22 must constitute an important part of the mAb 9.96 epitope. Although the limitations of this approach must be borne in mind, we feel that it may be of use in other fields of research.

The reactivity of the 17 kd E4 protein with all six anti-E4 antibodies indicates that this protein most probably represents the primary product of the HPV-1 E4 gene. Its reactivity with anti-E4 (spliced N terminus), but not with anti-E4 (unspliced N terminus) anti-peptide antibodies, suggests that the N terminus of this protein is derived from the E1 ORF, as predicted from mRNA studies (Chow *et al.*, 1987a,b). The ability of such an mRNA to encode a protein of 125 amino acids (containing five amino acids derived from E1 linked to 120 amino acids encoded by E4) with a mol. wt of 14 351, correlates well with its identification by SDS gel electrophoresis as a 17 kd polypeptide. In sections of HPV-1-induced papillomas, the 17 kd protein was found exclusively in cells below the stratum corneum and was present in the cytoplasmic inclusion granules characteristic of HPV-1-

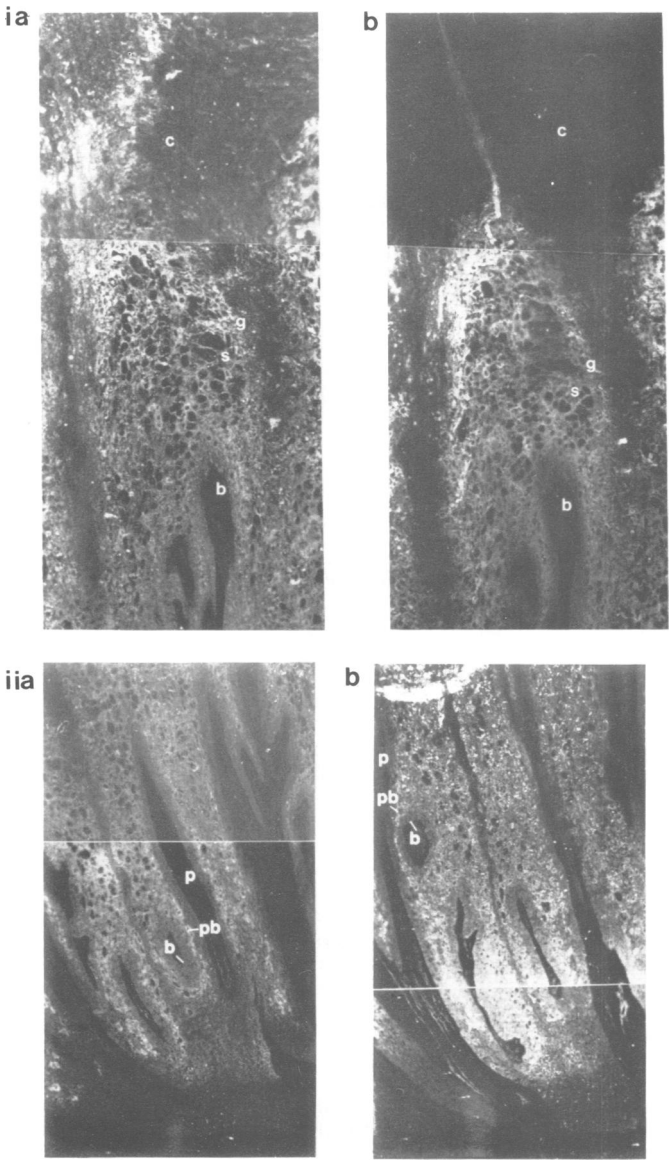

Fig. 9. Specific detection of the 17 kd (and 34 kd) E4 polypeptide in papilloma biopsies. (**i**) Detection of E4 proteins in the upper layers of HPV-1-induced wart following staining with either (**a**) mAb 4.37 or (**b**) anti-E4 (spliced N terminus) antiserum. The latter antiserum, which recognized only the 17 kd (and 34 kd) E4 protein, did not stain the upper cornified layer. b = basal layer, s = spinous layer, g = granular layer, c = cornified layer. Magnification = 80×. (**ii**) Typical E4 staining with both anti-E4 (spliced N terminus) (**a**) and mAb 4.37 (**b**) in lower layers of HPV-1-induced papilloma. b = basal layer, pb = parabasal layer, p = papilli. Magnification = 80×.

induced warts. This is in agreement with previous studies (Breitburd *et al.*, 1987) suggesting that the 17 kd polypeptide is the first E4 species to be detected in the deeper wart layers. It was surprising, however, to find that the ability to detect the 17 kd protein using anti-E4 (spliced N terminus) antibodies stopped quite suddenly and absolutely in cells of the upper spinous and granular layer. Although we cannot rule out the possibility that this is a result of a conformational change in the 17 kd protein, it seems more likely that partial degradation is responsible, in a manner analogous to that in which cytokeratins are degraded in the upper layers of skin epithelium (for review see Sun *et al.*, 1985). This is supported by the observation that the smaller E4 polypeptides (16 kd, 11 kd and 10 kd which may represent degradation or processed products of the 17 kd species), showed no reactivity against either the anti-E4 (spliced N terminus) or anti-E4 (unspliced N terminus) peptide antibodies, suggesting that they lack the reactive epitopes. The presence of the 16 kd protein (and possibly also the minor 21, 23 and 32 kd species) in the upper wart layers has previously been demonstrated (Breitburd *et al.*, 1987) and could be clearly shown using mAbs 4.37 or 9.95. If the 16 kd protein arises from the 17 kd as a result of N-terminal cleavage (as seems likely), then specific proteolysis must occur between the epitope sites recognized by anti-E4 (spliced N terminus) antibodies and mAb 7.76 (see Figure 7). It is an interesting observation that the leucine clusters, characteristic of the N-terminal E4 regions of all sequenced HPV types, are a common feature of other proteins which are known to be cleaved at their N terminus, where they form part of the eukaryotic signal sequence (for review see Warren, 1987).

If the 16 kd protein is derived from the 17 kd, then our results suggest that C-terminal sequences may also be partially degraded, as anti-E4 (C-terminal) antibodies which reacted strongly with the 17 kd proteins reacted only poorly with the 16 kd E4 protein. It should be borne in mind, however, that other explanations could be offered for our results, such as post-translational modification of the 16 kd protein, or its expression from a novel mRNA species. Conformational changes are unlikely to be involved, as binding studies were carried out against SDS denatured proteins.

The 10/11 kd E4 species appear to lack sequences derived from the 5′ end of the HPV-1 E4 gene, as determined by two different lines of study. It could not be recognized using three different mAbs directed against epitopes located in the N-terminal half of the E4 protein, and polyclonal antibodies prepared against purified 10/11 kd polypeptides showed reactivity only against bacterial E4 fusion proteins containing expressed regions derived from the 3′ half of the E4 gene. The reactivity of the 10/11 kd protein with mAb 8.40, but not with mAb 4.37, suggests that the E4 sequences it contains must begin between the epitopes recognized by these antibodies (amino acid 58–70, see Figure 2a). The amino acid sequence in this region is relatively well conserved between the E4 protein sequences of cutaneous papillomaviruses, and is rich in glutamic acid residues. This is most obvious in HPV-4, whose E4 gene encodes an unbroken string of 10 glutamic acid residues in this region (our unpublished results). It is possible that this sequence represents a specific proteolytic cleavage site within the larger E4 gene products. Alternatively, the smaller E4 proteins could be the product of a spliced mRNA containing only the central to 3′ end of the E4 gene. Analysis of mRNA species from cutaneous papillomas, however, has not revealed any such transcripts (Chow *et al.*, 1987a,b; Baker *et al.*, 1987) although a putative splice acceptor site exists within the E4 gene which could give rise to the 10/11 kd products (see Figure 7). Although we do not know the extent of the E4 sequences contained in the 10/11 kd proteins, their non-reactivity with anti-E4 (C terminus) antibodies suggests that they lack regions derived from the extreme 3′ end (as well as the 5′ half) of the E4 ORF. Our results, and the results of others indicating that the 10/11 kd proteins predominate in the upper cornified layers of HPV-1-induced warts (Breitburd *et al.*, 1987), may suggest that these species result from peptide processing. This is further suggested by the great

variability in the content of 10/11 kd proteins (but not other E4 species) in individual HPV-1 papilloma biopsies (Doorbar *et al.*, 1986).

We have previously reported that the 21/23 kd and 32/34 kd proteins may represent dimers of the 10/11 kd and 32/34 kd species respectively (Doorbar *et al.*, 1986; Breitburd *et al.*, 1987). Although our results indicate that the 16/17 kd and 32/34 kd gene products are related in this way, it seems unlikely that the 21/23 kd proteins are simple dimers of the smaller E4 proteins, as they consistently reacted with all three mAbs (7.76, 8.40, 9.60/9.95) which recognized epitopes in the N-terminal half of the E4 protein (i.e. those that showed no reactivity against the 10/11 kd protein). A number of mRNA species have been identified in HPV-1-induced papillomas which could give rise to E4 protein larger than 17 kd (Chow *et al.*, 1987a; see also Chow *et al.*, 1986). The exact origin of the 21/23 kd polypeptides, however, cannot be predicted at present, and it is possible that they arise either as a result of degradation of the larger 32/34 kd proteins or from polymerization of the 17 kd species with a smaller E4 gene product. If dimerization is involved, then some form of covalent bonding must be responsible such as that by which involucrin is cross-linked in the upper layers of normal epithelium (Watt and Green, 1981; Rothnagel and Rogers, 1984). It is interesting to note that previous studies using reactivity raised against bacterially expressed HPV-1 E6, E7, E1, E2, L2 and L1 ORFs did not identify the 21/23 kd protein (Doorbar *et al.*, 1986; Doorbar and Gallimore, 1987) suggesting that if splicing is involved, then it is likely to be to regions other than those expressed in the above mentioned fusion proteins.

Taken together, our results and those of other workers (Chow *et al.*, 1987b; Breitburd *et al.*, 1987) suggest that the 17 kd E4 protein is the product of an E1/E4 spliced mRNA, that is first expressed in the parabasal cells of HPV-1-induced warts. This most probably gives rise to proteins of mol. wt 16 kd and 10/11 kd in upper wart layers (Breitburd *et al.*, 1987). These species appear to lack N-terminal sequences present in the 17 kd polypeptide—the 16 kd lacking no more than ~15 amino acids, and the 10/11 kd lacking ~70 amino acids (Figure 7). Antibody studies suggest that the 32/34 kd polypeptides are 16/17 kd dimers, and we assume that the other high mol. wt proteins are E4 polymers. The 21/23 kd E4 gene products are not, however, simple dimers of the 10/11 kd proteins. It is not known whether the individual E4 species each have independent functions or whether E4 function is contained solely on the 17 kd primary gene product. In fact E4 function has yet to be established, although a number of suggestions have been made (Doorbar *et al.*, 1986; Neary *et al.*, 1987; Breitburd *et al.*, 1987). The determination of the relationship between the E4 proteins represents the first step towards analysing their function. In this respect, the specific reagents, and the monoclonal mapping technique described in this study, should prove useful in future research.

Materials and methods

Typing of warts

Total DNA was extracted from small pieces of individual wart biopsies as described previously (Doorbar *et al.*, 1986) and was typed either by agarose gel electrophoresis and Southern blotting or by dot blotting (Maniatis *et al.*, 1982). Probes were prepared by nick translation (Rigby *et al.*, 1977) of cloned HPV-1, HPV-2 or HPV-4 DNA (Burnett, 1983) using either biotin-11-dUTP or [^{32}P]dCTP. In the case of probes prepared using biotin-11-dUTP, hybrids were visualized using a commercially available system (BRL).

Purification of HPV-1 E4 protein and production of monoclonal antibodies

The HPV-1 16/17 kd E4 proteins were purified from an individual HPV-1-induced papilloma as previously described (Doorbar *et al.*, 1986), and were used to immunize three BALB/c mice. Individual animals were injected at multiple subcutaneous sites with 12 μg of 16/17 kd protein in Freund's complete adjuvant (day 0) and immunization was repeated after 14 and 28 days using the same amount of protein. Mice were test bled following the third immunization, and their anti-E4 antibody titres determined by ELISA. Animals showing a good response were given a final intraperitoneal (i.p.) injection containing 12 μg 16/17 kd protein in Freund's incomplete adjuvant, and their spleens were taken 3 days later. Spleen cells were fused with the mouse myeloma line X63-Ag8.653 as previously described by Rowe *et al.* (1982). Initial screening of hybridoma culture supernatants was performed by ELISA as described by Rowe *et al.* (1987) using purified 16/17 kd protein (100 ng/well) as target. Hybridomas of interest were recloned by limiting dilution, and characterized further by Western blotting. IgG typing was carried out using a commercially available immunodiffusion system (Seratec) and protein A binding determined by ELISA.

Preparation of E4 deletion plasmids

Fusion protein deletions were derived from plasmid p1p7 (Doorbar *et al.*, 1986)—a pEX1 (Stanley and Luzio, 1984) expression clone containing a 301 bp *Pst*I fragment (nt 3227–3529 on HPV-1 map) from the E4 ORF of HPV-1. After linearization with *Xba*I, 25 μg of p1p7 was treated with 1.5 units of exonuclease *Bal*31 as described previously (Maniatis *et al.*, 1982). Aliquots (20 μl) were taken at 2 min intervals (five time points) and the reactions were stopped by the addition of an equal volume of phenol (buffered in 50 mM Tris pH 8.0, 10 mM EDTA). Following precipitation by standard procedures (Maniatis *et al.*, 1982), the DNA was subsequently digested with *Eco*RV, and fragments were separated by preprarative agarose gel electrophoresis. The smaller fragment (which contained the deleted E4 gene region and which varied in size from 2.2 to 1.6 kb depending on the extent of *Bal*31 treatment) was eluted from the gel, end-filled with the Klenow fragment of *Escherichia coli* DNA polymerase (BCL) (Maniatis *et al.*, 1982) and cloned into pEX1, which had previously been digested with *Sma*I and *Eco*RV prior to being purified by agarose gel electrophoresis. The ligated DNA was used to transform competent *E.coli* POP 2136 cells (Raibaid *et al.*, 1984) as described by Hanahan (1983) except that all incubations were carried out at 28°C (heat shock at 32°C), and bacteria were pelleted at 4000 r.p.m. for 5 min instead of 2500 r.p.m. for 12 min. The colonies obtained were grown up, and their plasmid DNA was analysed by restriction enzyme digestion (Birnboim and Doly, 1979). Recombinants which contained inserts in the correct orientation were further checked by fusion protein expression as previously described (Doorbar *et al.*, 1986).

Determination of deletion size and DNA sequencing

E4 deletion constructs which contained correctly orientated fragments were double digested with *Hin*dIII and *Sma*I which cleaved the E4 region from the plasmid. After end-labelling the digested DNA with L-[^{35}S]dATP (Amersham International) using Klenow fragment of *E.coli* DNA polymerase (BCL) (Maniatis *et al.*, 1982), the size of the E4 insert was determined by separation on a 6% polyacrylamide DNA sequencing gel. M13 mp18 DNA was sequenced using the chain termination method (Sanger *et al.*, 1977), and was run alongside the labelled fragments as a size marker. Control DNA, containing inserts of known size (i.e. p1p7, pEX1) were also included to determine the accuracy of the technique.

The *Hin*dIII/*Eco*RV fragments from expression clones UUU, LLL, GGG, 1.102, A3, A22 and C27 were subcloned into M13 mp18 which had been previously digested with *Sma*I and *Hin*dIII, and treated with calf intestinal alkaline phosphatase (BCL). After transformation of competent *E.coli* JM101 (Messing *et al.*, 1981) single-stranded DNA was prepared as described by Winter and Fields (1980) and sequenced according to the method of Sanger *et al.* (1977) using L-[^{35}S]dATP (Amersham International).

Small scale preparation of fusion proteins and Western blotting

Bacteria containing recombinant plasmids were grown slowly overnight at 28°C in Eppendorf tubes containing 1.25 ml L-broth plus 25 μg/ml ampicillin. Care was taken to ensure that the culture did not reach an OD (500 nm) >0.4. Following induction at 42°C for 90 min (with vigorous agitation), the bacteria were pelleted (Eppendorf centrifuge, 2 min) and the pellet was solubilized by boiling for 5 min in 100 μl lysis buffer (5% SDS, 50 mM Tris–HCl, pH 8.0, 15 mM β-mercaptoethanol). Aliquots (10 μl) were mixed with an equal volume of sample buffer (2% SDS, 80 mM Tris–HCl,

pH 6.8, 10% glycerol, 0.025% BPB) and were analysed by SDS–polyacrylamide gel electrophoresis as described previously (Doorbar *et al.*, 1986). Western blotting was carried out as described by Towbin (1979) except that nitrocellulose filters were blocked in phosphate-buffered saline (pH 7.5) containing 0.5% gelatin. Antibody binding was detected using a commercially available peroxidase-linked streptavidin–biotin system (Amersham International).

Identification of specific proteins in wart biopsies

Total wart proteins were analysed by SDS and alkaline urea polyacrylamide gel electrophoresis as described elsewhere (Doorbar *et al.*, 1986). Indirect immunofluorescence was carried out either on acetone-fixed 5 μm frozen sections, or on paraffin embedded sections after fixation in either formalin: acetic acid:methanol (1:1:8) or in Bouin's fluid (17 parts picric acid, 2 parts formaldehyde, 1 part glacial acetic acid). FITC-Labelled goat anti-rat or anti-mouse IgG was used as the second antibody.

Preparation of anti-synthetic peptide antibodies

Synthetic peptides were produced either by the Macromolecular Analysis Service, University of Birmingham, or by the Molecular Immunology Laboratory, Imperial Cancer Research Fund Laboratory, London). After purification, N-terminal E4 peptides were linked via their cysteine residues to bovine thyroglobulin and bovine serum albumin using *N*-γ maleimidobutyryloxysuccinimide (Sigma) as described by Yoshitake *et al.* (1979), or to keyhole limpet haemocyanin and ovalbumin using glutaraldehyde (Guiso *et al.*, 1984) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimie (ECDI, Sigma) (Tamura *et al.*, 1983). The C-terminal peptide was linked via its cysteine residue to ovalbumin and bovine chymotrypsinogen as described above. Antibodies were prepared after immunization of hooded Lister or Lou rats as previously described (Doorbar *et al.*, 1986).

Computational analysis

HPV-1 E4 secondary structure and epitope prediction studies were performed on an IBM PC using the 'Protean I' protein analysis program (see Robson *et al.*, 1987; Proteus Biotechnology Limited, Poynton, Stockport, Cheshire, UK/Department of Biochemistry and Molecular Biology, University of Manchester, Manchester, UK). Hydrophilicity profiles were produced using a BBC model B microcomputer, according to the method of Hopps and Wood (1981).

Analysis of the EMBL, Doolittle and PIR data bases was carried out on the University of Birmingham Multics computer, running the Bristol University 'DNA sequence program' version 10.5.

Acknowledgements

We are greatly indebted to Margaret Gilbert, and the staff and students at the School of Chiropody (Mathew Boulton Technical College, Birmingham, UK), to members of the Birmingham Chiropody Society, and to Sister R. Meakin (Sutton Coldfield Cottage Hospital, Birmingham, UK) for collecting the biopsy samples used in this study. In this respect, we would also like to thank Dr Herbert Pfister and colleagues (Institut für Klinische Virologie, Erlange, FRG) for generously providing us with a considerable number of cutaneous warts, and to anyone we have failed to mention above who has been involved in obtaining clinical material used in this work. Thanks also to Barry Robson (Department of Biochemistry and Molecular Biology, University of Manchester, Manchester, UK) and Robert V.Fishleigh (Proteus Biotechnology, Poynton, Stockport, UK) for carrying out the Protean I epitope prediction analysis, and J.Rothbard (Molecular Immunology Laboratory, ICRF, London, UK) for preparing the N-terminal peptides. The expert technical assistance of Valerie Nash and Liz Fletcher, and the secretarial skills of Debbie Williams are gratefully acknowledged. This work was supported by the Cancer Research Campaign, UK. P.H.G. is a Cancer Research Campaign Life Fellow.

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Received on December 7, 1987