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Proteins Present in Bovine Papillomavirus Particles

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Analysis by two-dimensional gel electrophoresis and silver staining of heavy full, light full, and empty bovine papillomavirus particles has shown that the major capsid protein L1 is highly modified. Besides exhibiting at least 13 isoelectric point variants of approximately the same molecular mass (54 kilodaltons), it is suggested that an additional heavier protein chain (69 kilodaltons) is also derived from L1 by glycosylation. These modifications may stabilize the particle structure. Treatment with neuraminidase reduces the number of modification products detectable, with a concomitant increase in the more basic forms of L1. Although it was not possible to detect histones in any of the preparations, proteins of similar molecular mass were detected. Therefore, it is suggested that the basic tails of L1 bind to the DNA in a manner similar to that of histone. Calculation of the theoretical mobilities of the papillomavirus proteins shows good agreement with the actual position of L1 and its isoelectric point variants and suggests that two of the proteins with molecular masses similar to those of the histones may actually be coded by the bovine papillomavirus E7 and E5 open reading frames.

The papillomaviruses are becoming increasingly significant members of the DNA papova group of tumor viruses as it becomes evident that they can induce malignant conversion in the human host. It has been recognized for many years now that papillomaviruses can induce benign and malignant tumors in many animal species, including cottontail rabbit (25), cattle (1), dog (23), horse (11), sheep (28), elk (16), and mouse (19), but only relatively recently has the magnitude of human infections caused by them become evident.

Today there are at least 46 types of human papillomavirus (HPV) recognized; they are found in a wide variety of lesions from tongue papilloma, laryngeal papilloma, skin warts, epidermodysplasia verruciformis, and condyloma, several of which give rise to malignant tumors (4, 21). Although studies of the papillomavirus DNA have been making rapid progress, studies of the virally encoded proteins have been severely hampered by the absence of a permissive cell culture system, and it is becoming increasingly more urgent to identify these proteins and their functions in the cell.

One approach to studying the papillomavirus proteins has been to isolate the intact particles from infected tissue by differential and buoyant density gradient centrifugation (6, 8, 12, 13, 27). These studies resulted in the demonstration that the following three bands usually could be observed in CsCl gradients: heavy full particles (ρ = 1.36 g/ml), light full particles (ρ = 1.34 g/ml), and empty particles (ρ = 1.29 g/ml). The terms full or empty refer to the presence or absence of DNA, respectively.

In their rigorous exposition of virus structure, Klug and Finch (10) deduced from a meticulous analysis of electron microscopy pictures that the outer shell (capsid) of the papillomavirus particle has a skewed icosahedral surface lattice composed of 72 morphological units. This deduction was based in part upon the observation of both five- and six-fold axes of symmetry through different capsomeres, showing that the capsomeres occupied only quasiequivalent positions in the structure and that therefore they could not be identical. Although all the papillomavirus sequencing to date has found two so-called late open reading frames (L1 and L2), no one has been able to demonstrate the presence of two structural proteins in the viral capsid; this situation has given rise to an enigma in the literature which, as far as we are aware, no one has attempted to elucidate.

If the two proteins L1 and L2 are components of the capsid, then they would be expected to be found in the same ratio as the number of five- and six-fold axes (i.e., 12:60), and thus both should be easily detectable (26). However, the two proteins have similar theoretical molecular masses (55,556 and 50,020 daltons [Da], respectively, from the first methionine) and might be posttranslationally modified so that they become indistinguishable upon one-dimensional gel electrophoresis. All published reports indicate the existence of a single capsid protein with an estimated molecular mass of about 54 (6), 59 (12), 53.5 (15), and 56 kDa (9) for bovine papillomavirus (BPV), with similar values for HPV.

Additional proteins have been detected in the particles with a variety of molecular masses, but usually including three or four proteins migrating in a manner similar to that of the hosts' core histones, and have been suggested to be involved in packaging the DNA. Since these studies were performed, significant improvements have been made in both protein resolution, with the advent of high-resolution two-dimensional gel electrophoresis, and highly sensitive silver staining methods for detecting proteins.

Calculation of the theoretical isoelectric points for the two capsid proteins (L1 and L2) shows, however, that they will be resolved easily on two-dimensional gels.

Therefore, this study was undertaken to increase our knowledge of the components to be found in BPV particles.

MATERIALS AND METHODS

Isolation of BPV. The BPV particles were isolated from naturally occurring calf papillomas essentially by the method described by Meinke and Meinke (15). All procedures were performed at 4°C unless otherwise indicated. The papilloma tissue was minced, suspended in 6 volumes of 0.1 M Tris hydrochloride (pH 7.5)–0.01 M EDTA, and homogenized in an Ultra Turrax three times for 5 min each time with 5-min intervals for cooling. When required, the sample was di-

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gested with trypsin (final concentration, 0.01%) for 1 h at 37°C (22). The homogenate was centrifuged at 10,000 × *g* for 15 min, and the pellet was suspended in 5 volumes of 0.1 M Tris hydrochloride (pH 7.5)–0.25% Sarkosyl by using the Ultra Turrax. Subsequently, 5 volumes of 1,1,2-trichlorotrifluoroethane were added and vortexed at room temperature. The phases were separated by centrifugation at 3,000 × *g* at room temperature for 5 min, and the supernatant was removed and added to the previous supernatant. The remaining material usually consisted of a brownish pellet below a solid white layer.

Where indicated in Results, the pooled supernatants were sonicated twice for 3 min each time, with an amplitude of 10 μM (MSE Soniprep 150) at this stage.

The virus-containing supernatants were mixed with a stock CsCl solution (ρ = 1.33 g/ml), and the final density was adjusted to 1.31 g/ml as determined by refractive index. These samples were then centrifuged in a TST 41 rotor at 110,000 × *g* for 40 h at 25°C. After centrifugation, the tube was punctured at the bottom and 6-drop fractions were collected.

After samples were taken for electron microscopy, the amount of proteins present was determined (2) and the density gradient was measured (by refractive index). The virus-containing fractions were pooled appropriately and the virus was recovered by the addition of 3 volumes of ethanol at −20°C. This virus fraction was left to precipitate for a minimum of 2 h at −20°C and pelleted at 10,000 × *g* for 20 min. The pellet was washed three to four times with 70% ethanol at −20°C to remove the CsCl (again, the samples were left to precipitate for 2 h after each wash). The resulting virus pellet was freeze-dried overnight.

Treatment of virus particles with neuraminidase. The virus pellets were suspended in 0.1 M sodium phosphate buffer (pH 6.5)–20 mM EDTA, and 1 U of neuraminidase was added per 10 mg of protein and left to digest for 24 h at room temperature.

Treatment of virus particles with endoglycosidase F. The virus pellet was resuspended in 0.1 M sodium phosphate buffer (pH 6.1)–50 mM EDTA–1% Nonidet P-40–0.1% sodium lauryl sulfate–1% β-mercaptoethanol and boiled for 6 min before the addition of 4 U of endoglycosidase F per 10 mg of protein. Digestion was performed for 24 h at room temperature (24).

Two-dimensional gel electrophoresis. The procedures for two-dimensional gel electrophoresis were essentially those described by O'Farrell (19) for the isoelectric focusing (IEF) gels, by O'Farrell et al. (20) for the nonequilibrium pH gradient electrophoresis (NEPHGE) gels, and by Fey et al. (7) for the extended-range NEPHGE gels. Briefly, the first dimension gels were all 3.8% acrylamide–0.2% bisacrylamide and the pH gradient was stabilized by ampholytes in the following ranges: IEF, 3.5to 10 and 5 to 7 (LKB Instruments, Inc., and Serva); NEPHGE, 7 to 9 and 8 to 9.5 (LKB); and extended-range NEPHGE, 7 to 9, 8 to 9.5, and 9 to 11 (LKB). In each case, the actual mix and running time must be determined with each new batch. In all cases, the second dimension was run on 15% acrylamide–0.075% bisacrylamide gels (7, 17a).

Silver staining of polyacrylamide gels. The procedures for silver staining have been described previously (7, 17; P. M. Larsen, Cand. Scient. thesis, Aarhus University, Aarhus, Denmark, 1981).

RESULTS

Particles from several bovine papillomas were isolated by differential and buoyant density gradient centrifugation, and the proteins were extracted and analyzed by high-resolution two-dimensional gel electrophoresis. The gels were then stained by a highly sensitive silver-staining procedure which permits the detection of proteins in amounts as little as 1 ng, allowing the detection of minor components of the viral particles.

The densities of the three peaks A, B, and C obtained by CsCl buoyant density gradient centrifugation of crude virus preparation (Fig. 1) corresponded well with the previously reported value for papillomavirus full heavy (ρ = 1.36 g/ml), full light (ρ = 1.32 g/ml), and empty particles (i.e., particles devoid of the viral DNA genome; ρ = 1.29 g/ml) (22). In each of these fractions, the presence of viral particles (with suitable structure and diameter) was confirmed by electron microscopy (data not shown).

Two-dimensional gel electrophoresis (IEF and NEPHGE) of the proteins extracted from these three peaks revealed differences in the degree of modification of some of the proteins (Fig. 2A to C).

Photographs of the entire gels (both IEF and NEPHGE) are shown in Fig. 2B (light full particles), whereas only selected regions are shown from the heavy full (Fig. 2A) and empty particles (Fig. 2C). Several of the spots are impurities but serve as standards for comparison (labeled only in Fig. 2B). On the NEPHGE gel, the major contaminants were the immunoglobulin G (IgG) heavy chain group and IgG fragments (IgG'). These contaminants have molecular masses ranging from 52 to 57 kDa and 35 to 40 kDa, respectively,

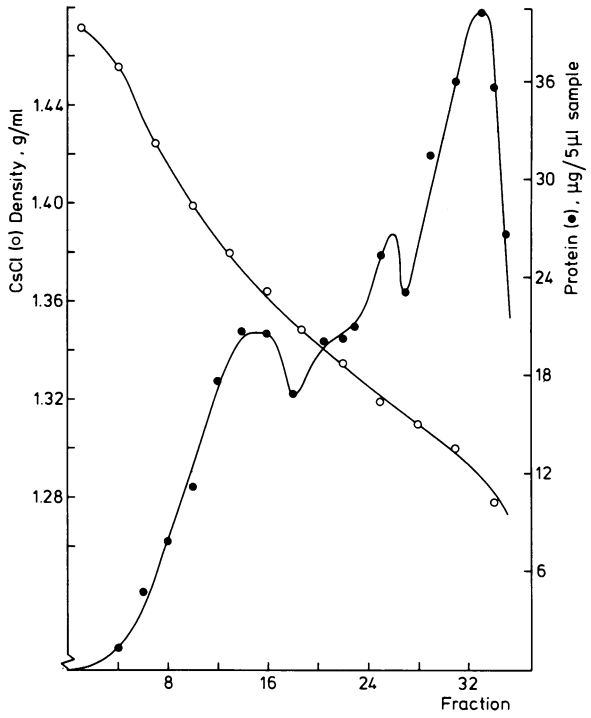

FIG. 1. Distribution of proteins after CsCl buoyant density centrifugation. A crude preparation of BPV particles prepared by homogenization and differential centrifugation was mixed with CsCl to a final density of 1.31 g/ml and centrifuged at 110,000 × *g* for 40 h at 25°C. The density (left axis) was determined by its refractive index, whereas the protein concentration (right axis) was determined by the method of Bradford (2). Fractions 9 to 17 were pooled and labeled heavy full particles (peak A), fractions, 20 to 25 were labeled light full particles (peak B), and fractions 29 to 33 were labeled empty particles (peak C).

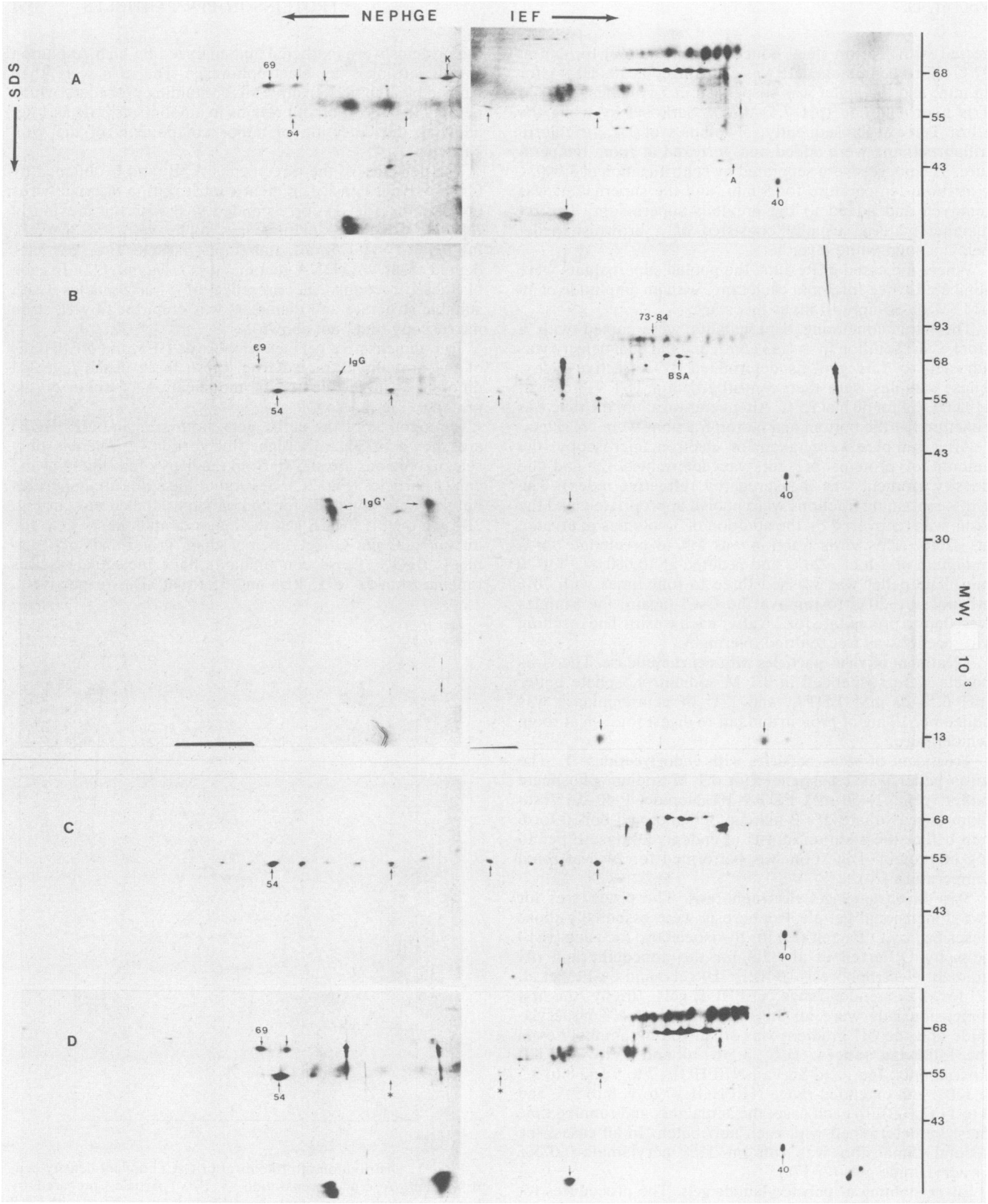

FIG. 2. Two-dimensional gel analysis of the proteins present in the BPV particles. The protein from the pools obtained as described in the legend to Fig. 1 was precipitated with alcohol and washed with 70% alcohol. After freeze-drying, the protein was run on two-dimensional gels (IEF and NEPHGE) and silver stained as described in Materials and Methods. Shown are the proteins in heavy full (A), light full (B), and empty particle pools (C). (D) A sample of the heavy full particles (10 mg) was treated with 1 U of *Clostridium perfringens* neuraminidase (Sigma Chemical Co.). The full two-dimensional gel is shown in panel B, whereas only a region is shown in panels A, C, and D. The numbers refer to the apparent molecular masses (right axis) determined by coelectrophoresis with [^{35}S]methionine-labeled total HeLa cell proteins Abbreviations: IgG and IgG′, immunoglobulin G heavy chain and its fragments; BSA, bovine serum albumin; SDS, sodium lauryl sulfate.

and coelectrophorese with these proteins from normal serum samples (i.e., mixtures of these samples with bovine serum resulted in no additional spots in this region of the gel, demonstrating the identity of the two proteins; data not shown).

On the IEF gel (Fig. 2B), the major contaminant is bovine serum albumin (molecular mass, 68 kDa), having the same spot configuration as and coelectrophoresing with the purified protein (data not shown). Above bovine serum albumin at 73 to 84 kDa on the IEF gels, there is an additional group of spots which exists in reasonable amounts in the heavy or light full particles but in only trace amounts in the empty particles. In some preparations from other bovine warts, this protein shows up to eight additional, more highly acidic, modification products (data not shown). The total amount of this protein varied so much from preparation to preparation that we considered it to be an impurity.

Trace amounts of some keratin proteins were also detected on the gels (Fig. 2, K), illustrating the degree of purification of the particles (keratins can comprise over 50% [by weight] of the total protein present in wart tissue). The keratin was identified by coelectrophoresis with crudely purified bovine keratin (data not shown). Occasionally, in electron micrographs small filaments were observed adhering to virus particles.

A small amount of actin is also visible in the IEF gels (43 kDa; Fig. 2A). Besides the IgG, IgG', keratin, bovine serum albumin, and actin, spots which are not referred to or which are not indicated with arrows were detectable in either bovine epithelial cells or plasma and therefore are considered to be contamination.

Repeated density gradient centrifugation resulted in the reduction of these contaminants and also in a reduction in degree of modification of some of the other proteins and so was not routinely performed. Sonication of the crude virus preparation also reduced the amount of these contaminants but in addition resulted in a much poorer yield of viral particles and so was not performed subsequently. Treatment of the crude homogenate with trypsin resulted in numerous additional spots which could not be identified in the original material and which thus were assumed to be degradation products. The keratins especially produced numerous ladders of spots which became more acidic as more residues were removed. Thus, trypsin was not used to help release virus particles. Therefore, all attempts to reduce the contamination led also to a reduction in the yield or quality of the particles, and so further reduction of contamination was not subsequently attempted.

The chain of spots at about 54 kDa most clearly visible in the empty particles (Fig. 2C) is considered to be the major viral protein L1. This result corresponds well with previously published values (12) and with the size of the protein predicted on the basis of the L1 open reading frame of BPV-1 (5). This is the first instance in which this protein has been shown to be modified. The range of isoelectric points for the different modification products spans from approximately pH 9.5 to pH 6.5, each succeeding spot (left to right) having gained one additional acidic group.

The actual amounts of the different modification products of L1 varied much more in the heavy full and light full particles (Fig. 2A and B). Those spots which existed in higher amounts in the gels of empty particle preparations also tended to be those found in the full particles. In addition to these spots, an extra short chain of spots was detected at about 69 kDa in the heavy full and light full particles which was not detected in the empty particles. Weak bands have been detected at this molecular mass on one-dimensional gels of purified particles before (12, 15), but they have not been investigated further. These spots cannot be contaminating keratin or its degradation products because both of these proteins are more acidic and have lower molecular masses.

Since their positions on the gels suggests that they might be related to the L1 protein by glycosylation, samples of the three peaks were treated with the enzyme neuraminidase, which removes the terminal sialic acid residue commonly found on glycoproteins. This treatment resulted in an increase in the amount of one of the least-charged spots at 54 kDa (Fig. 2D) and a corresponding decrease in the total amount of the other modification products; some modification products had been preferentially stripped (Fig. 2D, asterisk). Despite the use of a large excess of the enzyme it was not possible to strip all the modifications from the proteins. Digestion of similar viral pools with an excess of endoglycosidase F, an enzyme which completely removes both high-mannose and complex N-linked oligosaccharide side chains by cutting between the amino acid and the sugar, yielded similar results under denaturating conditions (data not shown).

Most previous reports describe a group of low-molecular-mass proteins which have approximately the same molecular mass as the calf core histones, and so, by analogy to simian virus 40, it has been suggested that BPV also uses the host's histones to pack its DNA (6, 12, 22). Since histones are not resolved by the normal NEPHGE two-dimensional gels shown in Fig. 2, it was necessary to run an extended-range NEPHGE gel. The proteins extracted from the heavy full and light full particle pools (A and B) were electrophoresed on the extended-range NEPHGE gels and showed, surprisingly, that no histones could be detected in either sample (Fig. 3A; only a region of the gel from the heavy full particle peak is shown). Gels loaded with five times the amount of protein shown in Fig. 3A still failed to show the histones. Bovine core histones (H2A, H2B, H3, and H4) migrated to the position shown in Fig. 3B, and coelectrophoresis of the histone and pool A confirmed their absence (Fig. 3C). Several other proteins exist in the purified full particles having similar molecular masses (Fig. 2B and 3A), and it seems possible that they were the proteins detected by other groups and described in previous publications. These proteins were absent from the empty particles (Fig. 3D), as was reported previously for the histonelike particles (6, 12, 22).

DISCUSSION

This study was initiated to increase our knowledge of the proteins present in viral particles.

The results obtained here by using silver-stained two-dimensional gels of virus preparations have demonstrated that the coat protein L1 is extensively modified and that L2 does not appear to be an integral part of the capsid.

In order to accommodate bonding to adjacent subunits in both five- and sixfold symmetries, the L1 molecules, if identical, would be conformationally stressed. The differential charge modification reported here would offer one possible way to reduce this stress and hence increase the stability of the particle.

Theoretical calculations predict values for the mobility of L1 and its modification products very close to those actually observed. The exact number of modification products detected in Fig. 2C is unknown. If the actual amounts form a repeating pattern of two modification products in smaller

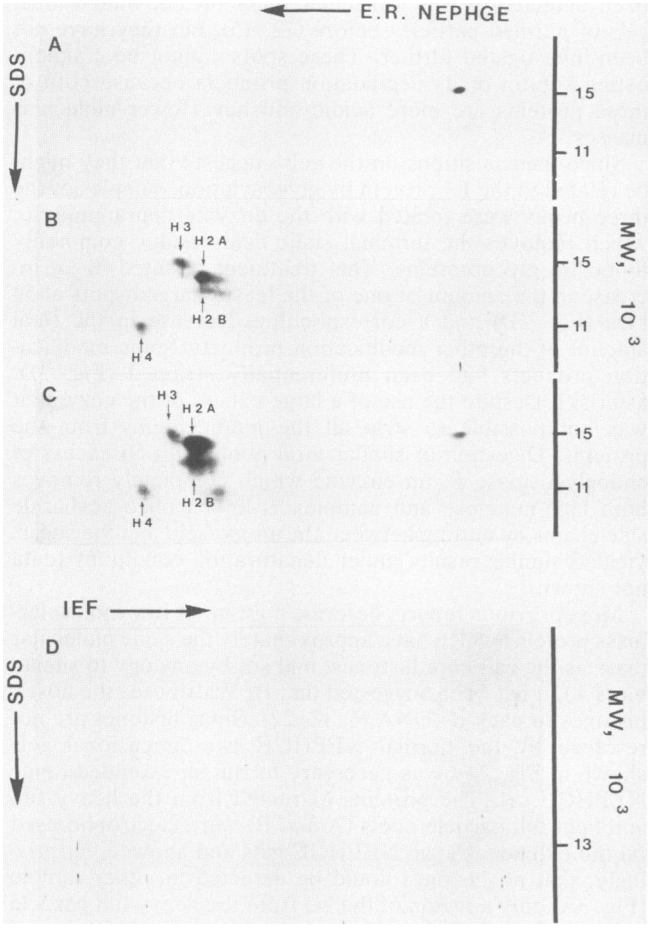

FIG. 3. Low-molecular-mass region of the silver-stained two-dimensional gels of proteins in BPV particles. Shown are proteins from the heavy full particle pool (A), purified calf thymus core histones (Sigma) (B), and coelectrophoresis of samples A and B analyzed by extended-range (E.R.) NEPHGE. (D) Absence of low-molecular-mass proteins from the empty particle pool as analyzed with IEF gels. SDS, Sodium lauryl sulfate.

amounts followed by two modification products in larger amounts, this pattern would suggest an overlap of one spot between the IEF and NEPHGE gels and would result in a total of 12 easily detectable modification products plus the original spot. Several of the modification products appear to be double spots, differing only slightly in their molecular masses (by about 500 to 1,000 Da) but having the same isoelectric point. This increase in molecular mass appears to be necessary for further charge modification of the protein because otherwise an increasing stack of spots would be seen with increasingly higher charge modifications. This increase is extremely unusual and most likely has some significance in the actual particle structure.

The detection of what appear to be glycosylated forms of L1 located mostly in the heavy full particles raises some interesting points. First, only the least charged modifications of the L1 appear to be further glycosylated to the 69-kDa variant, and the resultant products are quite specific, suggesting that the modification is carefully controlled (often glycosylation results in a chain of cloudy spots on two-dimensional gels and not in the defined spot observed in the present study). Second, in the heavy full particles, one-third to one-half of the L1 may exist in this heavier glycosylated form, and it may be this form that gives rise to the difference in buoyant density. Because it has not been possible to strip these proteins of their modification completely by using neuraminidase or endoglycosidase F, sedimentation of digested heavy full particles has not yet been attempted to test this suggestion. Finally, it is unknown whether the L1 is modified before or after DNA encapsulation. If it is modified after encapsulation, then the light full particles might represent an immature stage of particle production.

Two common types of BPV are known (types 1 and 2), and it was of interest to determine whether the modification of L1 was a general feature or whether it was restricted to a particular BPV type. Therefore, these experiments were repeated with papillomas from four different calves (from different herds) and in each case have resulted in the demonstration of a chain of spots at 54 kDa. In addition, crude preparations of papillomas from an additional three calves showed the same pattern. Therefore, the modifications are considered to represent a general structural feature of the papillomavirus particle, irrespective of virus type. Recently, similar observations have been made on particles isolated from human hand and foot warts (submitted for publication).

The absence of histones from the particles was quite surprising. In view of their proposed role in packaging the DNA, it appears unlikely that they could be lost easily during preparation (6, 12, 22). To package the 8 kilobases of BPV DNA into nucleosomes containing 200 base pairs each would require about 80 molecules of each histone. The sensitivity of the analysis used here would have allowed their detection even if only a few copies of each histone existed on average per particle, and so it must be concluded that they do not appear to be playing their conventional role. Therefore, the very basic tail of the theoretical L1 molecule, having 14 potentially negatively charged side groups in the last 33 amino acid residues, may play a role similar to that described for the basic tails of the histones in packaging the DNA. Such linking would be expected to contribute significantly to the stability of the DNA-containing viral particle. Differences in the stabilities of the particles have been described before (3). Evidence for this proposal has been published recently in a work which shows that L1 and some other papillomavirus proteins have DNA-binding activities (14). Therefore, the proteins which were reported by other groups to have mobilities similar to those of the histones on one-dimensional gels may correspond to the proteins observed in the present study which have similar molecular masses but very different isoelectric points.

Of the other proteins which appear to be enriched in the viral particles, the protein on the IEF gels that has a molecular mass of 40 kDa is of significance. Two proteins having mobilities very similar to those of this protein have also been identified in virus particle isolates from human hand and foot warts. By labeling these warts with [^{35}S]-methionine in vitro it was possible to demonstrate that these two proteins plus a third protein at 58 kDa could be immunoprecipitated with an antiserum produced against papillomavirus particles (submitted for publication); therefore, these proteins may be related by specific degradation. These proteins could be L2 on the basis of theoretical mobility calculations and are present in only a few copies per particle. The protein at 58 kDa is preferentially labeled in light full particles, and its degradation may play some part in the assembly or maturation of the particle.

Theoretical calculations based upon the open reading frames of the BPV-1 sequence suggest that the spot marked only with an arrow at 35 kDa in Fig. 2B may correspond to E2 and that the two low-molecular-mass spots at about 12 kDa may correspond to E5 and a modification product of it. Similar spots have also been detected in the HPV particles (submitted for publication). The single spot shown in Fig. 3A on the extended-range NEPHGE might correspond to E7. These proteins are repeatedly isolated with the viral particles, and so if they are not some impurities they may be either virally encoded (E2, E5, and E7) or used by the virus for some as yet unknown purpose. In either of the latter two cases it will be instructive to determine the function of these proteins, as well as of the other proteins of the BPV particle, in order to increase our understanding of the process of infection by papillomavirus.

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