Association of the Human Papillomavirus Type 11 E1-\textsuperscript{E4} Protein with Cornified Cell Envelopes Derived from Infected Genital Epithelium

Janine T. Bryan\textsuperscript{*} and Darron R. Brown\textsuperscript{*†‡}

\textsuperscript{*}Department of Medicine and \textsuperscript{†}Department of Microbiology and Immunology, Indiana University School of Medicine, and the \textsuperscript{‡}Roudebush Veterans Administration Hospital, Indianapolis, Indiana 46202

Received June 14, 2000; returned to author for revision August 7, 2000; accepted August 18, 2000

INTRODUCTION

Cornified cell envelopes (CCEs) isolated from HPV 11-infected epithelial tissues differ from those isolated from uninfected epithelial tissue in ways that could facilitate transmission of virus from person to person (Brown and Bryan, 2000). These abnormalities, including a thinner wall, increased fragility, and morphological alterations, can collectively be considered another cytopathic effect induced by HPV 11 infection. Our previous data indicate that loricrin, the most abundant CCE protein, is abnormally distributed in HPV 11-infected genital epithelium (Brown and Bryan, 2000). The mechanisms used by HPV 11 to induce abnormalities in CCEs are not known, but it is possible that viral proteins are involved.

The HPV 11 E1-\textsuperscript{E4} spliced transcript encodes an 11-kDa phosphoprotein of unknown function (Bryan et al., 2000; Nasseri et al., 1987). The HPV 11 E1-\textsuperscript{E4} protein self-oligomerizes through noncovalent bonding (Bryan et al., 1998). The bicistronic E1-\textsuperscript{E4}-L1 transcript of HPV 11 encodes both the E1-\textsuperscript{E4} protein and the L1 major capsid protein (Brown et al., 1996; Bryan et al., 2000). The HPV 11 E1-\textsuperscript{E4} protein is detected in differentiated keratinocytes in the cytoplasm, concentrated near the CCE, while the L1 protein is detected in nuclei, where virus assembly occurs (Brown et al., 1994). Colocalization of the two proteins in differentiated keratinocytes suggests a role for the E1-\textsuperscript{E4} protein in the late stages of the viral developmental cycle.

For the E1-\textsuperscript{E4} proteins of HPV types 1 and 16, in vitro studies indicated an interaction with intermediate filament proteins (Doorbar et al., 1991, 1996; Roberts et al., 1994, 1997). However, in vivo studies did not confirm an association with intermediate filaments (Doorbar et al., 1991, 1996; Roberts et al., 1994, 1997).

A possible role for the E1-\textsuperscript{E4} protein may involve association with the CCE. Such an association could contribute to the abnormalities of the CCE proteins isolated from HPV 11-infected genital epithelium. In this study, we analyzed HPV 11-infected genital tissues to determine whether CCE proteins were abnormally expressed in the cells containing detectable levels of E1-\textsuperscript{E4} protein. CCEs were then purified from infected genital epithelium, fragmented, and analyzed for evidence of association with the E1-\textsuperscript{E4} protein. Further studies were performed to examine the potential mechanisms of E1-\textsuperscript{E4} association with purified CCE fragments.
RESULTS

CCE proteins are abnormally distributed in E1^E4-positive cells

To correlate the distribution of CCE proteins with that of E1^E4 protein expression, histologic and immunohistochemical analyses were performed on foreskin tissue and genital lesions removed from three patients (Fig. 1). All three lesions contained histologic features of condyloma acuminata (Fig. 1E, shown for one of the three lesions) and abundant HPV 11 DNA, determined by a PCR/reverse blot strip assay (Gravitt et al., 2000).

Loricrin was detected in foreskin tissue as uniform cytoplasmic staining of differentiated keratinocytes (Fig. 1B). In condyloma acuminata lesions, loricrin was distributed unevenly in the differentiated layers of epithelium (Fig. 1F). Large areas of the lesions were devoid of loricrin, even in the most superficial layers of epithelium.

Cytokeratin 10 (K10) was detected in foreskin tissue as uniform staining of the cytoplasm of suprabasal keratinocytes (Fig. 1C). In condylomata acuminata lesions, K10 was distributed unevenly in the differentiated layers of epithelium, near the periphery of differentiated keratinocytes (Fig. 1G). As with loricrin, large areas of the lesions were devoid of K10, even in the most superficial layers of epithelium.

Involucrin was detected in the cytoplasm of suprabasal keratinocytes of foreskin tissue (Fig. 1D). Involucrin was detected in the cytoplasm of the suprabasal layers of epithelium in the HPV 11-infected condylomata acuminata lesions (Fig. 1H). Compared to uninfected epithelium, involucrin detection in the most differentiated layers was somewhat reduced.

Analysis of condylomata acuminata lesions showed that E1^E4 protein was concentrated near the periphery of differentiated keratinocytes, in the region of the CCE (Fig. 2). In tissues examined for CCE proteins, cells positive for E1^E4 protein were completely devoid of detectable loricrin (Fig. 3). Likewise, loricrin-positive cells contained no detectable E1^E4 protein. This mutual exclusion was regional, meaning that large areas of tissue contained either E1^E4-positive cells or loricrin-positive cells, but not both. The mutual exclusion was also present on a cell-by-cell basis. A mutual exclusion was also seen for K10 and E1^E4 protein (Fig. 4). All three condylomata acuminata lesions displayed mutual exclusion of loricrin (or K10) and E1^E4 protein (shown for one of the three lesions). No specific abnormalities of involucrin distribution were apparent in individual E1^E4-positive cells of the condylomata acuminata lesions (not shown).

The E1^E4 protein copurifies with CCEs

Athymic mouse xenografts of human foreskin provide a source of abundant HPV 11-infected tissue. CCEs were therefore isolated from athymic mouse xenografts as described under Materials and Methods. CCEs were therefore isolated from athymic mouse xenografts as described under Materials and Methods. Some were left intact (not sonicated). The remainder were fragmented by sonication. Phase-contrast microscopy showed that less than 1% remained intact after sonication (not shown). A portion of the CCE fragments was extracted using reducing agents. CCE preparations were then solubilized in Laemmli buffer (Laemmli, 1970) and separated by 15% SDS–PAGE.

Immunoblots were performed to determine whether the E1^E4 protein was present in CCE preparations. Abundant E1^E4 protein was detected in immunoblots of intact CCE preparations and in CCE fragments prepared without the use of reducing agents (Fig. 5, Lanes 1 and 2). A prominent doublet at 10/11 kDa was present, as well as faint bands at approximately 20 and 30 kDa. In contrast, no E1^E4 protein was detected in the immunoblot of CCE fragments prepared using reducing agents (Fig. 6, Lane 3).

Immunoblots were also performed on CCE fragments purified using reducing agents, followed by degradation with cyanogen bromide. Highly purified CCE fragments

---

are completely insoluble, but can be cleaved by cyanogen bromide to yield variable sized, soluble peptides derived from numerous CCE proteins (Yaffe et al., 1993). Cyanogen bromide cleaves proteins after methionine residues. The resulting range of soluble, cross-linked, and uncross-linked peptides can then be resolved by SDS–PAGE. Our initial analysis of cyanogen bromide-degraded CCE fragments was performed using 15% SDS–PAGE. However, proteins did not adequately resolve, and repeat experiments were therefore done using 10% SDS–PAGE.

CCE fragments prepared using reducing agents contained no soluble protein, as demonstrated on the silver-stained 10% SDS–PAGE gel (Fig. 6A, Lane 1). Therefore, as expected, the immunoblot of these CCE fragments was negative for E1°E4 protein (Fig. 6C, Lane 1). Cyanogen bromide degradation of these CCE fragments yielded a smear of multiple-sized peptides on the silver stain gel (Fig. 6A, Lane 2). Anti-E1°E4 immunoreactive peptides were detected throughout this broad smear (Fig. 6C, Lane 2). A covalent linkage of E1°E4 protein to the CCE protein matrix would predict such an immunoblot result. Yaffe et al. (1993) described a similar immunoreactive pattern for involucrin in cyanogen bromide-degraded CCEs. In addition to the broad immunoreactive smear, we noted two areas with enhanced staining at approximately 60 and 75 kDa in the blot probed with anti-E1°E4 serum (Fig. 6C, Lane 2). This observation suggested a concentration of E1°E4-linked peptides at these molecular masses. CCE fragments derived from uninfected genital epithelium showed no reactivity with preimmune rabbit serum or anti-E1°E4 serum (not shown). Immunoblots of CCE fragments prepared from HPV 11-infected genital epithelium using preimmune rabbit serum demonstrated no reactivity before or after cyanogen bromide degradation (Fig. 6B, Lanes 1 and 2).

The E1°E4 protein is present in purified CCE fragments

Immunofluorescence (IF) analysis using anti-involucrin and anti-E1°E4 sera showed that both proteins were present in CCE fragments derived from HPV 11-infected epithelium (Fig. 7). Involucrin was chosen as a control for identification of the CCE protein matrix in this assay because the immunohistochemical studies demonstrated generally uniform distribution in HPV 11-infected epithelium.
lium. Involucrin was identified in all CCE fragment preparations by IF, making involucrin a suitable control protein.

Anti-involucrin antibodies reacted well with CCE fragments from HPV 11-infected tissue, while only weak background green fluorescence was seen using preimmune rabbit serum (Figs. 7A and 7B). CCE fragments from HPV 11-infected tissues purified without the use of reducing agents fluoresced strongly with anti-involucrin and anti-E1\(^E4\) sera (Figs. 7C and 7D), while CCE fragment purification in the presence of reducing agents led to a moderate reduction of fluorescence with anti-E1\(^E4\) serum but consistent fluorescence with anti-involucrin antibodies.
(Figs. 7E and 7F). Weak background green fluorescence was seen in CCE fragments derived from uninfected epithelium using anti-E1\(^*\)E4 serum (not shown).

In the immunoelectron microscopy studies, numerous fields were examined for each antibody/CCE fragment combination. Two examples are shown in Fig. 8 for each combination. Involucrin was used as a control for CCE protein detection. This protein was identified in nearly all CCE fragment preparations by decoration with 5-nm gold beads (Fig. 8, all panels). Within each CCE fragment preparation, individual fragments displayed somewhat variable degrees of involucrin detection. This can be best seen in Fig. 8, Panels 2a and 2b, where little involucrin was detected in Panel 2a compared to Panel 2b.

For E1\(^*\)E4 protein detection, incubation with preimmune rabbit serum led to minimal decoration with 15-nm gold beads (Fig. 8, Panels 1a and 1b). E1\(^*\)E4 protein was detected in abundance in CCE fragments prepared without reducing agents (Fig. 8, Panels 2a and 2b) and in the presence of reducing agents (Fig. 8, Panels 3a and 3b). As mentioned above, Panels 3a and 3b illustrate two examples of the numerous fields examined. Our general impression was that E1\(^*\)E4 protein detection was somewhat reduced in CCE fragments prepared in the presence of reducing agents.

In some cases, decoration of CCE fragments was more or less than in others, but we made our conclusions only after examining numerous fields by EM. The significance of the study is not in the number of beads, but rather the detection of proteins based on the presence of beads. Our IEM study provides strong evidence

FIG. 5. Immunoblot analysis of intact CCEs and CCE fragments derived from HPV 11-infected athymic mouse foreskin xenografts. Anti-E1\(^*\)E4 serum was used to identify the E1\(^*\)E4 protein in intact (unsonicated) CCEs (Lane 1), CCE fragments extracted in 0.2% EBN (Lane 2), and CCE fragments extracted in 0.2% EB (containing reducing agents) (Lane 3). Molecular mass markers (in kDa) are shown on the left side of the figure.

FIG. 6. 10% SDS–PAGE/silver stain (A) and immunoblots (B, preimmune rabbit serum; C, anti-E1\(^*\)E4 serum) of proteins derived from CCE fragments purified using reducing agents. Lane 1, CCE fragments before degradation with cyanogen bromide. Lane 2, CCE fragments degraded with cyanogen bromide. Molecular mass markers (in kDa) are shown on the left side of the figure. Arrows in C indicate two areas of enhanced staining in the smear at approximately 60 and 75 kDa in the blot probed with anti-E1\(^*\)E4 serum, suggesting a concentration of E1\(^*\)E4-linked peptides at these molecular masses.

FIG. 8. Immunoelectron microscopic analysis of CCE fragments derived from HPV 11-infected athymic mouse foreskin xenografts. (1a/b) Two electron photomicrographs of CCE fragments purified with 0.2% EBN, incubated with mouse anti-involucrin antibodies and preimmune rabbit serum, and then decorated with 5-nm gold bead-labeled anti-mouse antibodies and 15-nm gold bead-labeled anti-rabbit antibodies. (2a/b) Two micrographs of CCE fragments purified with 0.2% EBN, incubated with mouse anti-involucrin antibodies and anti-E1\(^*\)E4 rabbit serum, and then decorated as above. (3a/b) Two electron photomicrographs of CCE fragments purified with 0.2% EBN, incubated with mouse anti-involucrin antibodies and anti-E1\(^*\)E4 rabbit serum, and then decorated as above. Original magnification, 34,000×.
that the E1\(^{+}\)E4 protein is present in CCE fragments that have been treated so vigorously that few, if any, soluble proteins remain. The conclusion is that E1\(^{+}\)E4 is cross-linked within the CCE.

**DISCUSSION**

Keratinocytes exit the cell cycle in the suprabasal layers of epithelium and undergo the process of differentiation that includes expression of protein components of the CCE (Rice and Green, 1977). The CCE consists of covalently cross-linked proteins in a sheath adjacent to the interior surface of the keratinocyte cell membrane (Nemes and Steinert, 1999). The end-point of differentiation and keratinization is a layer of flattened, enucleated CCEs, protecting against water loss, mechanical injury, and invasion by microbes. Analysis of CCEs has demonstrated the presence of loricrin, involucrin, K10, small proline-rich proteins, and other proteins covalently linked through transglutamination and disulfide bonds (Candi et al., 1995; Marvin et al., 1992; Ming et al., 1994; Robinson et al., 1997; Steinert et al., 1998; Steinert and Marekov, 1997; Yaffe et al., 1993).

Because HPV virions are assembled in nuclei of differentiated keratinocytes, a durable CCE would be seemingly detrimental to virion release. To spread virus to new hosts, a mechanism must exist for exiting the nucleus and the CCE structure. Our previous immunohistochemical analysis suggested that a profound alteration in loricrin distribution occurs in HPV 11-infected epithelium (Brown and Bryan, 2000). In the current study, we show that on a cell-by-cell basis, the detection of the E1\(^{+}\)E4 protein and the detection of loricrin, the major protein component of CCEs, are mutually exclusive. A similar mutual exclusion was noted for E1\(^{+}\)E4 and K10. Interestingly, abnormal distribution of cytokeratins has also been described for lesions infected with HPV types 1 and 16 in regions of E1\(^{+}\)E4 protein detection (Doobar et al., 1997). These authors did not perform studies for detection of loricrin, the major protein component of the CCE (Doobar et al., 1997).

It is possible that abnormalities of loricrin and K10 expression could contribute to the altered mechanical features of CCEs isolated from HPV 11-infected epithelium. CCEs from healthy human genital epithelium are composed of 65 to 70% loricrin (Steven and Steinert, 1994). Alterations in the expression pattern of loricrin in numerous dermatological disorders have been observed (Hohl, 1993). Keratin intermediate filaments represent a small percentage of CCE proteins, but may serve an important role as anchors to the CCE through isopeptide crosslinking (Candi et al., 1998; Ming et al., 1994). Involucrin did not appear to be distributed abnormally in HPV 11-infected tissue, although the dense staining in the most differentiated layers of healthy epithelium was not observed in the infected tissues. Additional CCE proteins such as the small proline-rich proteins may also display altered distribution in E1\(^{+}\)E4-positive cells. However, these possibilities have not been tested as we have not performed a comprehensive examination of CCE protein expression in HPV 11-infected epithelium. The reasons for the mutual exclusion of E1\(^{+}\)E4 with loricrin and K10 proteins are not apparent, but studies are under way to determine the mechanisms involved.

This is the first demonstration of an association of the E1\(^{+}\)E4 protein with any keratinocyte structure in vivo. The function of the E1\(^{+}\)E4 protein has not been determined, and our study does not prove that the HPV 11 E1\(^{+}\)E4 protein causes abnormalities of CCEs. Several previous reports have shown that the E1\(^{+}\)E4 proteins of HPV types 1 and 16 associate with the keratinocyte intermediate filament network of undifferentiated keratinocytes when expressed in vitro and cause disruption of the network in some cases (Doobar et al., 1991; Roberts et al., 1994, 1997). In contrast to the in vitro experiments, Doorbar et al. (1996) showed that the HPV 1 E1\(^{+}\)E4 protein does not associate with intermediate filaments in natural plantar wart tissue.

Disulfide bonding plays an important role in formation of the CCE (Candi et al., 1999; Hohl et al., 1991) and may be important in the association of E1\(^{+}\)E4 protein with the CCE structure. As the keratinization process occurs, disulfide bonds are formed from free sulfhydryl groups at the interface of the living layers of epithelium with the stratum corneum (Yamada et al., 1987). The activity of the enzyme responsible for this process, sulfhydryl oxidase, is concentrated in the stratum granulosum (Yamada et al., 1987). In this epithelial layer, development of the CCE begins, and the E1\(^{+}\)E4 protein is first detected. It is therefore possible that E1\(^{+}\)E4 protein is linked to CCE components by the action of sulfhydryl oxidase in the stratum granulosum. Our data support the hypothesis that at least a portion of E1\(^{+}\)E4 protein association with CCEs occurs through disulfide bonding.

In summary, profound abnormalities of expression of two CCE proteins, loricrin and K10, were observed in E1\(^{+}\)E4-expressing cells in HPV 11-infected epithelium. Purification of CCEs from infected tissue revealed an association of E1\(^{+}\)E4 protein with the CCE structure. Disulfide bonding appears to be important in this association. Further studies will characterize the direct effects of E1\(^{+}\)E4 protein expression on the CCE using in vitro models.

**MATERIALS AND METHODS**

**Immunohistochemical analysis of epithelial tissue for CCE proteins**

Human genital epithelium was analyzed by immunohistochemistry for the presence and distribution of E1\(^{+}\)E4 and three CCE proteins. The tissues examined included three condylomata acuminata lesions, all containing abundant HPV 11 as determined by a PCR/reverse blot strip assay (Brown et al., 1999; Gravitt et al., 2000). Zinc formalin-fixed paraffin-embedded sections were depar-
affinized with xylene and then with ethanol and water. One section from each biopsy was stained with hematoxylin and eosin for examination of tissue histology. Additional sections were used in immunohistochemical assays. The antibodies used were a rabbit polyclonal antiserum against a 14-amino-acid peptide found in loricrin (BAbCo, Richmond, CA), mouse monoclonal antibodies against involucrin and K10 (Novacastra, Newcastle upon Tyne, UK), and a rabbit polyclonal antiserum against a bacterially expressed HPV 11 trpE-E1^{4}E4 fusion protein (Brown et al., 1994).

To detect E1^{4}E4 protein, involucrin, and K10 in tissues, the “Microwave Calibration Protocol” was used with the Antigen Retrieval Citra Solution (BioGenex, San Ramon, CA). Following this procedure, slides were incubated in 0.1% trypsin in Tris–HCl, pH 7.8, with 0.1% CaCl₂ for 5 min. These steps were not used for detection of loricrin, as they did not enhance detection. Primary antibodies were added at a dilution of 1:500 for 16 h at room temperature. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used to detect antibody binding to E1^{4}E4 and CCE proteins according to the manufacturer’s instructions. Areas of anti-E1^{4}E4 antibody binding, detected using Vectastain VIP, were identified by light microscopy as purple staining of differentiated keratinocytes. CCE proteins, detected by Vectastain DAB, were identified as brown staining of suprabasal keratinocytes.

**Preparation of purified CCEs from epithelial tissue**

The athymic mouse xenograft system provides a source of abundant HPV 11-infected epithelial tissue from which CCEs can be purified (Brown and Bryan, 2000). Foreskin tissue was infected with purified HPV 11, implanted, and removed from mice 90 days after implantation (Brown et al., 1994; Kreider et al., 1987). Control xenografts were not infected with HPV 11, but were otherwise treated in an identical manner.

Implant tissue was minced using a scalpel and then extracted three times for 15 min at 100°C in 0.2% extraction buffer (0.2% EBN) containing 100 mM Tris–HCl, pH 8.0, 0.2% SDS, and 5 mM EDTA, but no reducing agents. Pelleted material was suspended in 500 μl of 0.2% EBN and then layered onto 3% Ficoll in 0.2% EBN, CCEs were separated from large tissue fragments by centrifugation at 1000 g for 15 min at room temperature. CCEs were visible in the gradient as a sharp white band and were collected, pelleted, and washed three times with 0.2% EBN. The presence of intact CCEs was verified by phase-contrast microscopy. CCEs were suspended in 0.2% EBN and stored at 4°C.

Purified, intact CCEs have been shown to contain abundant proteins not directly associated with the CCE structure (Yaffe et al., 1993). To produce CCE fragments, a portion of CCEs derived from uninfected and HPV 11-infected tissue was sonicated twice for 60 s using a Fisher Sonic Dismembrator (Model 300) with the intermediate size tip, at a setting of 60% of maximum output. Sonicated CCE fragments were examined by phase-contrast microscopy to ensure that less than 1% remained intact. CCE fragments were pelleted and washed with 0.2% EBN three times. A portion of CCE fragments was then extracted two additional times as above in 0.2% E8, containing 100 mM Tris–HCl, pH 8.0, 0.2% SDS, 5 mM EDTA, 10 mM DTT, and 130 mM β-mercaptoethanol. The pelleted CCE fragments were washed with 0.2% EBN to remove reducing agents, resuspended in 0.2% EBN, and stored at 4°C.

**Immunoblot analysis of CCEs for E1^{4}E4 protein**

Intact CCEs and CCE fragments (prepared with and without reducing agents) were pelleted, and an equal pellet size of each preparation (approximately 20 μl) was heated to 100°C in Laemmli buffer. Samples were briefly centrifuged to pellet insoluble proteins, and the soluble proteins were applied to 15% SDS–PAGE gels. Immunoblots were performed as previously described (Brown et al., 1992), using preimmune rabbit serum or anti-E1^{4}E4 serum.

**Digestion of CCEs with cyanogen bromide**

Cyanogen bromide cleaves proteins, including interlinked proteins found in CCEs, at methionine residues, yielding detectable quantities of soluble protein fragments in immunoblots (Yaffe et al., 1993). CCE fragments purified using reducing agents were suspended in 10% formic acid with 5% by weight, cyanogen bromide. Digestion was performed at room temperature, in the dark for 72 h. Reactions were quenched with 5 vol of water, and samples were desiccated. Samples were heated to 100°C in Laemmli buffer, and undigested insoluble CCE fragments were removed by centrifugation. Supernatants, containing digested CCE peptides, were analyzed by 15% and 10% SDS–PAGE and immunoblots as described above. Silver stains were performed using the Silver Stain Plus kit from Bio-Rad Laboratories (Hercules, CA).

**Immunofluorescence and immunoelectron microscopy of CCEs**

CCE fragments derived from HPV 11-infected human epithelium or uninfected human epithelium were prepared as above and washed three times in PBS-T (PBS containing 0.01% Tween 20) and then incubated for 2 h in 1 × Diluent/Blocker (Kirkegaard and Perry) at 4°C. CCE fragments were washed in PBS-T and then incubated in 1 × Diluent/Blocker containing either preimmune rabbit serum or anti-E1^{4}E4 serum at a 1:500 dilution. In addition, anti-involucrin monoclonal antibodies were added to each CCE preparation at a dilution of 1:100. Antibodies were removed by washing CCE fragments in PBS-T three times, and secondary antibodies were added. For immunofluorescence studies, a goat anti-rabbit FITC conjugate (Dako Corp., Carpinteria, CA) and a goat anti-mouse...
rhodamine conjugate (Roche Diagnostics Corp., Indianapolis, IN) were added at a dilution of 1:250. For immunoelectron microscopy studies, goat anti-rabbit (labeled with gold beads, 15 nm) or goat anti-mouse (labeled with gold beads, 5 nm) were added at a 1:60 dilution (Ameri-
sham Pharmacia Biotech, Inc., Piscataway, NJ). Secondary antibodies were incubated with CCE fragments for 16 h at 4°C, and unbound antibodies were removed by washing three times in PBS-T.

CCE fragments were examined at 488- and 590-nm wavelengths for immunofluorescence studies. For immunoelectron microscopy, CCE fragments were suspended in 3% gluteraldehyde prior to fixation and sectioning. Transmission electron micrographs were taken at a magnification of 34,000×.

ACKNOWLEDGMENTS

A Merit Review Award from the Veterans Administration supported this study. We thank Mr. Michael Goheen for his instruction and assistance in electron microscopy.

REFERENCES


