Immortalization and Altered Differentiation of Human Keratinocytes In Vitro by the E6 and E7 Open Reading Frames of Human Papillomavirus Type 18

JOHN B. HUDSON,¹ MARY A. BEDELL,² DENNIS J. McCANCE,³ AND LAIMONIS A. LAIMINS^{1,2*}

Howard Hughes Medical Institute¹ and Department of Molecular Genetics and Cell Biology,² The University of Chicago, Chicago, Illinois 60637, and Department of Microbiology and Immunology, University of Rochester, Rochester, New York 14642³

Received 18 August 1989/Accepted 23 October 1989

The E6-E7 region of human papillomavirus types 16 and 18 is selectively retained and expressed in cervical carcinoma cells. In cultured human keratinocytes, expression of the E6 and E7 open reading frames of human papillomavirus type 18, under the control of its homologous promoter, resulted in high-frequency immortalization. Furthermore, by using a system that allows for stratification of keratinocytes in vitro (raft system), we observed that the morphological differentiation of these E6-E7 immortalized cells was altered such that parabasal cells extended throughout most of the epithelium, with abnormal nuclei present in the upper regions. Examination of E6-E7-expressing cell lines in the raft system at a later passage revealed that complete loss of morphological differentiation had occurred. E7 alone was a much less effective immortalizing agent than E6 and E7 together and acted only minimally to alter morphological differentiation in vitro. No such activities were found for E6 alone. High-frequency transformation of human epithelial cells thus appears to require expression of both E6 and E7 gene products.

The probable etiological agents of human cervical and penile cancers are members of the papillomavirus family. Human papillomavirus (HPV) types 16, 18, and 31 are found in over 90% of cervical cancers, implicating them as etiological agents in at least the initial developmental stages of genital malignancies (9, 16, 25, 29). In contrast, the principal causative agent of benign genital warts, HPV-6 (29, 45), is only rarely found in malignant lesions. Epidemiological evidence suggests that infection by HPV-16, HPV-18, or HPV-31 alone may not be sufficient for progression to malignancy but that secondary factors may be required for progression of the disease (27, 41, 45).

Premalignant disease of the cervix (cervical intraepithelial neoplasia; CIN) is graded I (mild) through III (severe), depending on the extent to which the normal pattern of differentiation has been disrupted (10, 27, 45). Interestingly, the physical state of HPV sequences in these lesions often varies depending on the severity of the disease. In low-grade neoplasias, HPV genomes are often found as episomes which express a wide spectrum of early gene products. In contrast, in some high-grade premalignant lesions and in most cell lines derived from invasive cervical cancers, the viral genomes are found integrated into the host chromosomes (17). Integration of the viral genome into the host chromosome often occurs so as to disrupt E2 regulation of early gene expression (11, 13, 19, 36, 40), while the E6-E7 region is selectively retained and expressed in carcinoma cells (4, 35, 37, 39).

Previous in vitro studies with rodent cells have suggested that both of the E6 and E7 open reading frames (ORFs) of HPV-16 and HPV-18 encode independent transforming functions (7, 21, 22, 30, 42). For HPV-18, the E7 gene product alone was found to possess a strong transforming function in immortalized rodent fibroblasts, while the E6 ORF encodes a weak activity in these cells (7, 8). However, the combina-

tion of E6 and E7 was found to be most effective for transformation. Additional studies with both HPV-16 and HPV-18 have shown that E7 alone is sufficient for immortalization of primary rodent fibroblasts (7, 22) and that in cooperation with an activated *ras* gene, the E7 gene product can transform these cells to anchorage independence (7, 22, 26, 30). When WI38 human fibroblasts were used as recipient cells for HPV-16 transfections, the combination of E6 and E7 was found to be required for transformation to anchorage independence (43).

The natural host cells for HPV infection are stratifying epithelial cells, and recent studies have demonstrated that both HPV-16 and HPV-18 sequences are capable of immortalizing primary human keratinocytes in vitro (15, 23, 28, 31, 34, 44). We have previously shown that the entire HPV-16 genome can alter morphological differentiation in vitro in a manner similar to that seen in biopsies of HPV-infected genital lesions in vivo (28). By using the raft system, which allows for stratification and differentiation of epithelial cells in vitro, we observed morphological changes in an HPV-16-transfected cell line at low passage which resembled histological cross-sections of biopsies from low-grade neoplasias (28). Upon continued passage in culture, complete loss of differentiation was observed in this cell line, with a morphology similar to that observed in biopsies from high-grade neoplasias. In addition, when cell lines derived from biopsies of CIN grade I or III lesions were examined in the raft system, the morphology of the stratified epithelium bore a close resemblance to the biopsy from which the cells were derived (J. Rader, T. Golub, J. Hudson, M. Bedell, D. Patel, and L. Laimins, submitted for publication). These results suggest that the raft system is capable of accurately duplicating in vitro the state of in vivo lesions.

In the present study, we examined the intact E6-E7 region of HPV-18, as well as individual E6 and E7 gene products, for the abilities to immortalize keratinocytes and alter their differentiation in vitro. By using these two parameters as

* Corresponding author.

FIG. 1. Genomic organization and partial restriction map of HPV-18 (12). Early (□) and late (■) ORFs are shown. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; P, *Pst*I; S, *Sph*I; T, *Taq*I; X, *Xba*I. Also shown is the start of transcription for the E6-E7 region (arrow) and E6*, a spliced product of E6. Plasmid p18-1.5 reconstructs the early region of HPV-18 from the original *Eco*RI insert (8) and has 1.5 times the entire HPV-18 genome. p18PEpolyA contains a 3.4-kb *Pst*I-*Eco*RI fragment of HPV-18 inserted into pML and a 2-kb *Eco*RI insert from pSV2cat that contains the SV40 late polyadenylation signal (pA) (8). Translation termination linkers (TTL) were inserted into the *Bam*HI and *Hinc*II sites of p18PEpolyA to generate p18PEBam and p18PEHinc, respectively.

criteria for transformation, we found that both E6 and E7 are required for high-frequency transformation of human epithelial cells. At a reduced frequency, the E7 gene product alone also appeared to be capable of immortalizing primary epithelial cells but had only slight effects on their differentiation capabilities in vitro. These studies suggest a possible synergism of both the E6 and E7 gene products in the development of cervical intraepithelial neoplasias.

MATERIALS AND METHODS

Plasmids. The construction of all HPV-containing plasmids has been described previously (7, 8). Plasmids containing genomic (p18-1.5) or subgenomic (p18PEpolyA, p18PEBam, and p18PEHinc) portions of HPV-18 are shown in Fig. 1. Vectors which express either the HPV-18 E6 (p18MTE6) or E7 (p18MTE7) ORF from the human metallothionein promoter have been previously described (7). Either pRSVneo or pSV2hygro (provided by I. Blitz) was used for drug selection.

Culture of cells. Human keratinocytes were cultured from neonatal foreskin explants and used for electroporation after one or two passages. Cells were grown either in E medium with mitomycin C-treated 3T3 feeders (32) or in KGM (Keratinocyte Growth Medium; Clonetics Corp.). Identical results were obtained when primary cultures were propagated in either medium.

HPV-18-containing plasmids were linearized in plasmid sequences and combined at either a 10:1 or a 4:1 ratio with the selectable marker (either pRSVneo or pSV2hygro) to a total of 12 µg. Electroporations on 10^6 cells were performed as suggested by the manufacturer (Bio-Rad Laboratories) at 1,000 V and 25 µF in phosphate-buffered saline. At 2 days postelectroporation, selection for either G418 resistance (25 µg/ml) or hygromycin resistance (20 µg/ml) was applied for a

total of 10 days. No significant difference in number of colonies or efficiency of selection was observed with either drug selection method. Individual colonies were cloned and expanded or 5 to 20 colonies were combined into a mass culture in these studies. Cell lines were passaged once a week at a split ratio of 1:10 for immortalization studies and examined periodically in the raft system. Senescence of primary human epithelial cells was typically observed at four to five passages with this method.

Construction and subsequent manipulations of collagen rafts were performed as previously described (3, 24, 28). Keratinocytes were seeded onto collagen plugs containing 3T3 feeders. After cells reached confluence, the collagen was lifted onto a metal grid and cells were allowed to stratify at the air-liquid interface. After 14 days of culture on the metal grids, the collagen rafts were harvested for histological analysis (28). Transformed and normal cell lines were examined in the raft system at 5 or 10% fetal bovine serum for each lot of serum used. Different concentrations of serum were required for similar stratification of rafts, depending on the lot number. Southern and Northern (RNA) analyses were performed on cell lines as previously described (8).

RESULTS

HPV-16 and HPV-18 have been shown to be capable of immortalizing primary human epithelial cells. In a previous study, when a keratinocyte cell line containing transfected copies of HPV-16 was allowed to stratify in vitro in the raft system, an altered morphology of differentiation distinct from that seen with primary human keratinocytes was observed (28). In the present study, we first examined whether the entire HPV-18 genome contained on plasmid p18-1.5 (Fig. 1) was sufficient to immortalize and alter morphological differentiation in vitro in the raft system. Rafts exhibiting

FIG. 2. Hematoxylin- and eosin-stained rafts of keratinocytes with or without transfected HPV-18 sequences (A to C). Panels: A, keratinocyte cell line (150) containing p18-1.5 immediately following selection; B, keratinocyte cell line (154) immortalized by p18-1.5 examined at passage 20 in culture; C, neonatal foreskin keratinocytes on collagen rafts at passage 3; D, CIN grade III biopsy (magnification, $\times 165$).

any of the following features were considered abnormal in morphological differentiation *in vitro*: (i) lack of polarity in stratifying cells, (ii) disorganization or cell crowding in the parabasal layer, (iii) presence of mitotic figures in the upper half of the epithelium, (iv) increased nuclear to cytoplasmic ratio, or (v) abnormal nuclear appearance as exhibited by aberrant shape and pyknotic or enlarged pale nuclei. These features are often seen in cervical intraepithelial neoplasias *in vivo*. Cell lines immortalized by p18-1.5 were examined in the raft system, and two representative examples are shown in Fig. 2A and B. Cell line 150 (Fig. 2A), examined immediately after selection but before crisis, exhibited some disorganization and cell crowding in the parabasal region. However, a distinct granular layer, indicative of differentiation, was present. Cell lines containing p18-1.5 examined at a later passage (passage 20) showed a more altered pattern of differentiation with increased parabasal crowding and nuclear abnormalities, such as binucleation and aberrantly shaped nuclei in the upper regions of the epithelium (Fig. 2B). For comparison, a stratified raft of human neonatal foreskin keratinocytes exhibiting normal differentiation is shown in Fig. 2C. As an example of complete inhibition of differentiation, a cross-section of a CIN grade III biopsy is shown in Fig. 2D. Propagation of cell lines derived from CIN grade III lesions in the raft system shows a morphology similar to that seen *in vivo* (Rader et al., submitted). In our studies, cell lines containing the entire HPV-18 genome had a moderately altered pattern of differentiation.

We next examined whether the E6-E7 region alone, under the control of its own promoter, was sufficient to immortalize human keratinocytes and alter differentiation. Plasmid

p18PEpolyA (Fig. 1) contains the HPV-18 upstream regulatory sequences, intact E6 and E7 ORFs, a truncated E1 ORF, and a heterologous polyadenylation signal from the simian virus 40 (SV40) late region. In addition, this plasmid has previously been shown to be an efficient transforming agent of rodent fibroblasts *in vitro* (8). In this study, the p18PEpolyA plasmid was coelectroporated with either a pRSVneo or a pSV2hygro plasmid into passage 2 human keratinocytes. Following electroporation and drug selection, eight individual colonies were isolated and expanded. In addition, approximately 20 individual colonies were combined and examined as a separate mass culture.

Seven of eight p18PEpolyA-containing individual cell lines, as well as the mass culture, survived over 25 continuous passages and thus exhibited an extended life span in culture. In contrast, human keratinocytes typically senesce after four or five passages in culture when these techniques are used. We therefore conclude that both the E6-E7 region and the entire viral genome are efficient immortalizing agents of human keratinocytes, in agreement with previous studies (23, 44). None of the p18PEpolyA- or p18-1.5-containing electroporated cell lines tested induced tumors in nude mice (data not shown).

We examined the p18PEpolyA lines by Southern analysis for the presence of HPV-18 DNA, and all contained integrated copies of viral sequences at one to five copies per cell (data not shown). In all of the cell lines, the HPV sequences and the downstream SV40 polyadenylation signals were intact. Northern analysis of six individual p18PEpolyA cell lines and a mass culture confirmed that all lines expressed the E6-E7 region (Fig. 3A), and two classes of transcripts

FIG. 3. Expression of E6-E7 sequences in electroporated primary human keratinocytes. Total RNA was isolated from six individual p18PEpolyA-transfected cell lines, a mass culture, and the Bam-3 cell line transfected with p18PEBam. (A) Northern blot examined with an E6-E7-specific probe (720-base-pair *Bam*HI-*Taq*I fragment; Fig. 1). Lanes: 1, PE-1; 2, PE-2; 3, PE-3; 4, PE-4; 5, PE-5; 6, PE-6; 7, PE-mass; 8, Bam-3; 9, untransfected keratinocytes. (B) Same blot reprobed with an E1-specific probe (710-base-pair *Xba*I-*Eco*RI fragment from the E1 ORF; Fig. 1). The marks to the left of the panels indicate the positions of 18S and 28S rRNAs.

predominated. In four cell lines (PE-1, PE-2, PE-4, and PE-6), a 3.2-kilobase (kb) transcript was observed, while in two other cell lines (PE-3 and PE-5), a 1.4-kb transcript appeared to be the major transcript. However, most p18PEpolyA-derived cell lines express transcripts of both sizes, with one transcript size predominant in a particular clone. The mass culture appeared to express approximately equal amounts of each transcript size. Examination of these Northern blots with probes for the distal 3' end of the truncated E1 ORF (Fig. 3B) suggested that the 3.2-kb transcript probably encodes an unspliced transcript which uses the SV40 late poly(A) signal for termination, while the 1.4-kb transcript either terminates at the beginning of E1 or is a spliced product with a splice donor sequence located at the beginning of the E1 ORF.

When p18PEpolyA cell lines were examined for the ability to differentiate in the raft system, pronounced alteration of differentiation was observed, even at an early passage. A mass culture of p18PEpolyA-containing colonies was examined in the raft system, and a cross-section of a typical stratified epithelium after seven passages is shown in Fig. 4A. A disorganized parabasal layer was present throughout the lower half of the epithelium with an increased nuclear to cytoplasmic ratio and mitotic figures present in the upper half of the epithelium. In normal epithelium, dividing cells were limited to a single basal cell layer (Fig. 2C). The p18PEpolyA mass line still exhibited a polarity of stratification at this passage, with some degree of differentiation in the upper third of the raft. Three clonal cell lines (PE-2, PE-5, and PE-1) were also examined in the raft system at passage 7, and histological sections of these rafts are shown in Fig. 4C, E, and G. The alteration of morphological differentiation in all three cell lines was similar to that of the p18PEpolyA mass culture, but variations in the extent of alteration were observed among the cell lines.

When the mass culture and the individual clonal cell lines were examined at a later passage (passage 22) in the raft system, more complete disruption of differentiation was observed. In the mass culture (Fig. 4B) and clones PE-2 (Fig. 4D) and PE-5 (Fig. 4F), the cells at the top of the epithelium morphologically resembled those found in the basal layer. Similar results were found with most other clonal cell lines. We therefore conclude that passaging of most p18PEpolyA-containing cell lines in culture also contributes to loss of

their ability to differentiate. Only one clonal cell line, PE-1 (Fig. 4H), retained some ability to differentiate, as evidenced by the presence of a well-defined basal layer, but many aberrant and multinucleated cells were present in the upper regions of the stratified epithelium. By Northern analysis, the PE-1 cell line expressed the E6-E7 region at a lower level than the other cell lines (Fig. 3A, lane 1), but we do not know whether this low expression was responsible for the apparent reduced disruption of differentiation.

We next determined which of the E6 and E7 ORFs encodes the ability to immortalize and alter differentiation of keratinocytes. Passage 2 human neonatal keratinocytes were electroporated with plasmids containing translation termination mutations in either E6 (p18PEBam) or E7 (p18PEHinc) (Fig. 1) together with a hygromycin selectable marker, and individual colonies were selected. Six individual clones and a mass culture were expanded and examined for immortalization properties. In three experiments, all of the cell lines electroporated with p18PEHinc senesced at the same passage as controls electroporated with plasmids pUC9 and pSV2hygro (passage 4 or 5; Table 1). In contrast, cell lines electroporated with p18PEBam continued to be passaged for extended periods before senescence. While one p18PEBam-carrying individual clone (Bam-1) and one mass cell line (Bam-2) were passaged for 8 and 12 passages, respectively, before senescence, an additional mass cell line (Bam-3) survived to become an immortalized cell line (Table 1). Northern analysis of the Bam-3 cell line demonstrated that the E6 and E7 regions were expressed (Fig. 3, lane 8) but at a level reduced from that of most p18PEpolyA cell lines. Whether this reduction in expression was due to absence of the E6 gene product is unclear. We concluded that both E6 and E7 are required for high-frequency immortalization of primary keratinocytes, while E7 alone is able to extend the life of primary keratinocytes and, at a low frequency, provide for immortalization.

In support of the finding that E7 alone can extend the life span of primary keratinocytes, 2 of 12 cell lines that express the E7 ORF from a heterologous human metallothionein promoter (p18MTE7) survived 10 passages in culture (Table 1), while their parent cells senesced at passage 5 (data not shown). An analogous construct that expresses the E6 ORF (p18MTE6) failed to extend the life span of electroporated keratinocytes (Table 1).

The Bam-1, Bam-2, and Bam-3 cell lines were examined for the ability to differentiate in the raft system after passage 5 but before senescence. A representative Bam-1 raft is shown in Fig. 5A at passage 7, and a Bam-3 raft is shown in Fig. 5B at passage 12. In both cases, stratification with only slightly altered morphological differentiation was observed. Good polarity of differentiation was evident, with the presence of a distinct granular layer. In addition, no mitotic figures or abnormal nuclei were found in the upper regions of the epithelium. In contrast to the p18PEpolyA lines, it appears that passaging of the cell lines that express E7 alone may not significantly modify their differentiation properties in vitro. These studies suggest that while E7 alone can extend the life span of keratinocytes and immortalize cells at a low frequency, it has minimal effect upon epithelial differentiation. Maximal inhibition of differentiation, however, occurs when both the E6 and E7 ORFs are expressed.

DISCUSSION

In this study, we examined the ability of the E6-E7 region of HPV-18 to immortalize and alter the differentiation capa-

FIG. 4. Hematoxylin- and eosin-stained rafts of several p18PEpolyA-containing individual lines and a mass culture following stratification in the raft system. The cell lines were examined at passages 7 (lanes A, C, E, and G) and 22 (lanes B, D, F, and H). Panels: A and B, PE-mass; C and D, PE-2; E and F, PE-5; G and H, PE-1.

TABLE 1. Immortalization and extended life span of cells electroporated with plasmids containing mutations in the E6-E7 region of HPV-18 or expression vectors for the HPV-18 E6 and E7 ORFs

Plasmid	Promoter	ORF(s)	Clonal cell lines ^a	Mass cultures ^b
p18PEpolyA	HPV-18	E6*, E6, E7	7/8	3/3
p18PEBam	HPV-18	E7	1/7 ^c	2/3 ^d
p18PEHinc	HPV-18	E6, E6*	0/6	0/3
p18MTE7	HuMTII _A	E7	2/12 ^e	1/1 ^f
p18MTE6	HuMTII _A	E6, E6*	0/12	0/1
pUC9	None	None	0/12	0/2

^a Each plasmid (Fig. 1) was coelectroporated with a selectable marker into human keratinocytes. After selection for drug resistance, individual colonies were chosen at random, expanded into cell lines, and passaged weekly. Cell lines that survived more than 20 passages in culture were considered immortalized.

^b Five to twenty colonies were combined into mass cultures and examined as described for individual colonies.

^c The Bam-1 line senesced at passage 8.

^d The Bam-2 line senesced at passage 12; the Bam-3 line appeared to be immortalized.

^e The MT-2 and MT-3 lines senesced at passage 15.

^f The MT-1 line senesced at passage 12.

bilities of keratinocytes in vitro. While the E7 ORF alone can extend the life span of keratinocytes for several generations in vitro, expression of both E6 and E7 is required for high-frequency immortalization of keratinocytes. At an early passage, the combination of both E6 and E7 gene products altered differentiation in a manner histologically similar to

FIG. 5. Hematoxylin- and eosin-stained stratified rafts of cell lines that express the E7 ORF alone. Panels: A, Bam-1; B, Bam-3. The Bam-1 cell line was examined at passage 7, and it senesced at passage 8. The Bam-3 cell line was examined at passage 12 and appeared to be immortalized.

that seen in a CIN grade I or II lesion. Upon further passaging, the ability to differentiate was completely lost and the E6-E7-expressing cell lines had the histological appearance of high-grade CIN III lesions. Expression of the E7 ORF alone, even at a later passage (passage 12), had minimal effect on differentiation in vitro and resulted in morphologies consistent with normal epithelial differentiation. A reduced level of expression of E7 was seen in a cell line containing E7 alone (Bam-3), but whether this reduction was due to lack of a functional E6 protein is unresolved. The E6 gene product has previously been suggested to be a transcriptional activator of HPV-18 expression (19). Expression of E6 alone does not appear to be sufficient to immortalize keratinocytes, and we were unable to determine what, if any, effects this gene product had on differentiation when expressed by itself.

In human epithelial cells, both E6 and E7 appear to be required for high-frequency immortalization. This is similar to results of experiments performed in human fibroblasts (43) but in contrast to those of studies performed with rodent fibroblasts in which a single gene was found to be sufficient for transformation (7, 21, 22, 30, 42). Thus, while studies with rodent cells are informative, they may not accurately mimic the situation in human keratinocytes, the natural host cells for HPV infection. Furthermore, the continuous presence of E6 and E7 in cervical tumor lines suggests that these gene products play a role in oncogenesis in vivo (1, 5, 35, 38). In our studies, the E6-E7 region alone, under the control of its own promoter, altered the morphological differentiation of keratinocytes in the raft system to a greater extent than did the entire viral genome. We have previously suggested that this region alone is a more potent transforming agent than the entire virus and that integration in vivo leads to preferential expression of this region (8). One model suggests that this occurs by removal of a *cis* or *trans* repressor (8, 11, 13, 40). Complete analysis of this hypothesis will be facilitated by establishment of cell lines with episomal copies of HPV in which progression of the disease can be monitored.

In previous experiments using human keratinocytes transfected with HPV-16 DNA, we observed that the ability to differentiate decreased with further passaging (28). This was also observed with p18PEpolyA-carrying cell lines upon passage in culture, with more severe loss of differentiation capability at higher passages. Thus, it is important to monitor cell lines in the raft system as a function of passage number to accurately monitor the degree to which differentiation is altered by transfected gene products. The changes seen upon passaging are in agreement with the hypothesis that secondary cellular events contribute to the total loss of differentiation seen in HPV-18-induced neoplasias. Whether consistent chromosomal aberrations are associated with the total loss of differentiation remains to be examined. The finding that HPV-16 and HPV-18 can immortalize and alter the differentiation of keratinocytes in vitro, while no such activities have been demonstrated for HPV-6 (44), suggests that these studies reflect virus-cell interactions that occur in vivo.

The sequence analysis of the E6 and E7 ORFs of all papillomaviruses shows that the two gene products may have arisen by a duplication (12), and so it is not surprising that both contribute to epithelial cell transformation. The E6 and E7 proteins are localized to the nucleus (33), while a significant portion of E6 protein is also found in the cytoplasm (1, 2, 6, 19a). Both proteins contain cysteine doublets (Cys-X-X-Cys) (12) and have been shown to bind zinc (6; Grossman and Laimins, in press). The E6 protein also binds

DNA with high affinity but in a nonspecific manner (20). The biochemical characterization of these proteins will facilitate the understanding of how these proteins transform epithelial cells. In our studies, the E7 gene product alone extended the life span of keratinocytes but failed to alter their differentiation pattern. The large T antigen of SV40 can also immortalize keratinocytes and has minimal effect on differentiation (M. Lechner and L. A. Laimins, in preparation). It has been suggested that T antigen and E7 act through a common mechanism which involves physical interaction with the retinoblastoma gene product (14, 18). Our studies suggest that the binding and presumed resultant inhibition of retinoblastoma protein activity is probably not sufficient to inhibit differentiation and transform epithelial cells. Instead, the main target for the action of E7 and retinoblastoma proteins may be the control of cellular proliferation. We suspect that additional cellular targets for E6 and E7 action exist in transformed keratinocytes.

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