厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

ウイルスを標的とする発がん予防の研究

研究分担者　近藤　一成　NTT東日本関東病院　産婦人科医師

研究要旨
子宮頸癌はHPV感染によって引き起こされる。よってHPV感染を予防できれば子宮頸癌を予防できる。しかし現行ワクチンは16, 18型のみを標的にしているためにその他の型には有効でない。我が国のHPV型感染実態の把握が非常に重要と考えられている。

A．研究目的
子宮頸がんの原因はHPV感染である。HPVは100種類以上の型があると言われているが、がんに関わるのは15種類程度である。HPV感染を予防するワクチンが開発され市販化されているが、16型と18型HPVにしか有効でない。型分布には地域差があるために、現行のワクチンの効果も地域によって異なると考えられている。そこで、日本における正確なHPV型の感染実態を把握することを目的とした。

B．研究方法
NTT東日本関東病院にて産婦人科外来を受診した患者さんに説明を行い、同意が得られた人に対して子宮頚部の擦過細胞を回収した。細胞は専用の容器に入れ保管し国立感染症研究所村山分室に送り採取から2週間以内にDNAを抽出し保管した。PGMY法によるPCRを行い型特異的プローブによる型判定をした。
（倫理面への配慮）
当院倫理委員会の承認を得た。研究検査への協力に際して、説明書と同意書を用意した。また、十分な説明を行い、本人の直筆による同意書を頂いた。測定検体は匿名IDにより区別し、個人情報の秘匿化を実施した。

C．研究結果
438サンプルを解析した。このうち細胞診にて異常(日母分類のクラスⅢ以上)のあったサンプルは85例あり、80例(94%)がHPV陽性だった。30例(29%)は2種類以上の複数型感染であった。最も多かったのは58型(20例)で52型(18例)、16型(14例)、31型(7例)、56型(7例)、18型(5例)、51型(5例)と続いた。HSIL症例は30例あったが、16型(9例)、52型(9例)、58型(9例)だった。18型はなかった。子宮頸癌症例は13例あったが、16型(6例)、18型(2例)、52型(2例)、31型、82型がそれぞれ1例で陰性が1例あった。16型と53型の複数型感染が1例認められた。良性疾患の陽性率は以下の通りだった。妊娠初診(5/29:17%)、子宮筋腫(3/31:9.6%)、卵巣嚢腫(2/13:15%)、検診希望(0/9:0%)だった。

D．考察
子宮頸癌症例は症例数が少なかった為に正確な評価はできないが、16型、18型の頻度(61%)は欧米よりやや低めであった。症例数を増やして評価する必要性があると思われた。欧米におけるHSIL症例の型分布は16型が48%、31型が8%、33型と58型が7%、18型が6%、52型が5%と報告されている(Clifford et al, Vaccine 24S3 2006)が、日本では52型、58型が30%程度で検出された。これは今までにも指摘されてきたようにアジア地域の52、58型感染の多さを示しているものと思われる。頚部細胞診の異常のない人のHPV陽性率はおよそ10%前後であり今までの報告と同じであった。

E．結論
症例数は少ないものの、日本におけるHPV感染の実態を評価できた。特に52型や58型の感染が多いことが判明した。今までは複数型感染の率は10%前後という報告があるが、約30%位は複数型感染をしている事が判明した。PGMY PCRが感度よく様々な型を検出できる系であることが証明できた。

G．研究発表
1.　論文発表
なし
2.　学会発表
なし

H．知的財産権の出願・登録状況
（予定を含む。）
1. 特許取得
なし
2. 実用新案登録
なし

3. その他
なし

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分担研究報告書

ウイルスを標的にした発がん予防の研究

研究分担者　松本　光司　筑波大学 講師

研究要旨：子宮頸部前癌病変から検出されるHPV16のE6領域の変異を調べると、25番目のアミノ酸配列の変異によってその後の病変の自然消退の頻度に違いが見られることがわかった。HPVは変異によって宿主免疫から排除されにくい仕組みを持っている可能性がある。

A．研究目的

子宮頸癌患者から最も検出頻度が高いHPV16型のわずかな変異（variation）が前癌病変の自然消退や進展と関連があるかどうか調べること。

B．研究方法

多施設共同で行われた大規模コホート研究から、HPV16型陽性の前癌病変LSILを有する患者43例についてHPV16 E6領域のDNA変異をダイレクト・シーケンス法で調べ、病変の自然消退・進展との関連をKaplan-Meier法で解析した。

（倫理面への配慮）

研究実施計画書（プロトコール）のIRB承認を得た。すべての患者からインフォームドコンセントを文書で得た。データの取り扱いでは、匿名化して個人情報の保護を厳守した。

C．研究結果

E6領域のDNA配列の変異から推測されるE6タンパクのアミノ酸配列の変異のうち、25番目のアミノ酸がAspである患者（n=10）ではGlu である患者（n=33）よりも有意に病変が消失しにくかったが（12ヶ月後の病変消失率; 24% versus 72%, P=0.01）、病変の進展とは関連が見られなかった（P=0.24）。そのほかに、病変の自然消退・進展と関連のある変異は見られなかった。

D．考察

HPVは変異によって宿主免疫から排除されにくい仕組みを持っている可能性がある。免疫細胞へウイルス抗原の提示を行うHLA分子との組み合わせによって持続感染のリスクが異なるのかもしれない。今後はウイルス変異とHLAタイプとの関連についての解析が必要である。

E．結論

HPV16陽性前癌病変の自然消退とE6領域の変異には有意な関連が認められ、HPVは変異によって宿主免疫から排除されにくい仕組みを持っている可能性がある。

F．健康危険情報

G．研究発表

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2.　学会発表

H．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

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Nuclear location of minor capsid protein L2 is required for expression of a reporter plasmid packaged in HPV51 pseudovirions

Kazunari Kondo [a,b], Yoshiyuki Ishii [a], Seiichiro Mori [a], Shiho Shimabukuro [a], Hiroyuki Yoshikawa [b], Tadahito Kanda [a,*]

[a] Center for Pathogen Genomics, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Obstetrics and Gynecology, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba-shi, Ibaraki 305-8575, Japan

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ABSTRACT

The deduced amino acid (*aa*) sequence of L2 of the newly sequenced HPV51 strain, isolated by Matsukura and Sugase (Ma-strain), was markedly different from that of the prototype HPV51 isolated by Nuovo et al. (Nu-strain) (GenBank M62877) in two regions: *aa* 95–99 (region I) and *aa* 179–186 (region II). The two regions of Ma-strain were homologous to those of the other mucosal HPVs. The *aa* sequences of the N-terminal and C-terminal regions of Ma-L2 and Nu-L2 were identical and contained the nuclear localizing signal (NLS). When expressed in HEK293 cells, Ma-strain L2 (Ma-L2) was located in the nucleus but Nu-strain L2 (Nu-L2), in the cytoplasm. The chimeric L2s having both Nu-L2 regions I and II were located in the cytoplasm, and those having one of them were located both in the nucleus and cytoplasm, suggesting that Nu-L2 regions I and II inhibit the NLS function. For a better understanding of a role of L2 in infection, pseudovirion (PV) preparations were produced with a reporter, Ma-strain L1, and various L2s (Ma-L2, Nu-L2, or the chimeric L2s). These PV preparations contained structurally similar particles composed of L1 and L2 and the packaged reporter plasmid at a similar level. The reporter expression was not induced in HEK293 cells after inoculation with PVs containing the L2s that are incapable of localizing in the nucleus when expressed alone. Among PVs containing L2s capable of localizing in the nucleus, the reporter expression was induced only by PVs containing Ma-L2 region I. Thus, the results indicate that the expression of the reporter in the HPV51 PV requires the nuclear localizing ability of L2 and another unknown function associated with region I.

Introduction

Human papillomavirus (HPV) is a small nonenveloped virus with circular double-stranded DNA genome (8000 base-pairs) packaged in an icosahedral capsid composed of two capsid proteins L1 (major) and L2 (minor) (Howley and Lowy, 2001). Various HPVs, which have been identified in proliferative lesions including cervical cancer, are classified into more than 100 genotypes based on the homology of nucleotide sequence of L1 gene (Howley and Lowy, 2001). HPV infects the epithelium and propagates in the differentiating keratinocytes or establishes a latent state in the epithelial basal cells (Howley and Lowy, 2001). There are no cell cultures supporting efficient HPV propagation.

Infectious HPV pseudovirions have been developed (Unckell et al., 1997; Stauffer et al., 1998; Buck et al., 2004) and are used as a surrogate virus to study biological functions and immunogenicity of the capsid proteins (Sapp and Selinka, 2005; Buck et al., 2005). The mRNAs transcribed from the codon-modified L1 and L2 genes escape from the mechanism inducing the rapid degradation of the authentic L1- and L2-mRNAs in the undifferentiated cells (Sokolowski et al., 1998; Collier et al., 2002). L1 and L2 expressed in cultured cells from the codon-modified genes self-assemble to form L1/L2 virus-like particles (L1/L2-VLPs) in the nucleus. When L1 and L2 are expressed in the SV40 T-antigen positive cells transfected with a reporter plasmid carrying SV40-ori, the replicating reporter plasmid is packaged into the L1/L2-VLP to produce a pseudovirion (PV) (Buck et al., 2004). Because the efficiency of the packaging is not high, a semi-purified PV preparation contains complete PVs and empty L1/L2-VLPs. The reporter is expressed at a readily detectable level in the cells inoculated with the PV preparation.

According to the registered nucleotide sequence of HPV51 DNA (GenBank M62877), isolated from cervical condyloma by Nuovo et al. (1988) and sequenced by Lungu et al. (1991), the deduced amino acid (*aa*) of the HPV51 L2 is markedly different from those of the other mucosal HPVs in two regions, *aa* 95–99 (region I) and *aa* 179–186 (region II) (Fig. 1A). This led us to newly sequence the L2 gene of another HPV51 isolated from a Japanese woman with CIN (Matsukura and Sugase, 1995). In this study, we found that the two regions of the new isolate were different from those of the prototype but homologous to those of the other mucosal HPVs. We prepared and

* Corresponding author.
E-mail address: kanda@nih.go.jp (T. Kanda).

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A

	Region I	Region II
	aa95	aa179
HPV16L2	DPVGP	GHFTLSSST
HPV18L2	EPVGP	GNVFVGTPT
HPV31L2	DPVGP	GHILLSSSS
HPV52L2	EPIGP	GHVLFSSPT
HPV58L2	DTVGP	GHLIFSSPT
HPV51L2(Nu)	DLWHH	-DIYLLVHY
HPV51L2(Ma)	EPIAP	GHIFTSTLT

B

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Nu-L2    1 MVATRARRRKRASVTQLYSTCKAAGTCPPDVVNKVEGTTLADKILQWSGLGIFLGGLGIG  60
Ma-L2      ..............................I.............................

Nu-L2   61 TGSGSGGRTGYIPLGGGGRPGVVDIAPARPPIIIDLWHHTEPSIVNLVEDSSIIQSGSPI 120
Ma-L2      ..............................V..EPIAP.........D............

Nu-L2  121 PTFTGTDGFEITSSSTTTPAVLDITPSAGTVHVSSTNIENPLYIEPPSIEAPQSGEVS-D 179
Ma-L2      ...................................................GH

Nu-L2  180 IYLLVHYSGTHGYEEIPMEVFASNVSTGTEPISSTPTPGVSRIAAPRLYSKSYTQVKVTN 239
Ma-L2      .FTSTLT.....................................................

Nu-L2  240 PDFISKPSTFVTFNNPAFEPIDTSITFEEPDAVAPDPDFLDIITLHRPALTSRRGTVRFS 299
Ma-L2      ..........................N.................................

Nu-L2  300 RLGQKATMRTRSGKQIGARVHYYHDISRIAPADELEMQPLLSPSNNYSYDIYADLDEAET 359
Ma-L2      .....................................................

Nu-L2  360 GFIQPTHTTPMSHSSLSRQLPSLSSSMSSSYANVTIPFSTTYSVPIHTGPDVVLPTSPTV 419
Ma-L2      ..............V.....................P.....................

Nu-L2  420 WPYVPHTSIDTKHSIVILGGDYYLWPYTHLLRKRRKRIPYFFTDGIVAH 468
Ma-L2      ..................................................
```

Fig. 1. Amino acid sequence of L2. (A) Amino acid sequences of L2 *aa* 95–99 (region I) and *aa* 179–186 (region II) of prevalent mucosal HPV L2s. HPV51 isolated by Nuovo et al. (1988) (Nu-strain) is indicated as HPV51-Nu and HPV51 isolated by Matsukura and Sugase (1995) (Ma-strain) is indicated as HPV51-Ma. (B) Total amino acid sequences of L2 of the two HPV51 strains. The upper line (Nu-L2) shows amino acid sequence of L2 of HPV51 Nu-strain (deduced from the nucleotides sequences of GenBank M62877). The lower line (Ma-L2) shows that of HPV51 Ma-strain.

characterized PVs by using L2s of the two strains. We found that the reporter expression from HPV51 PVs appears to require two L2 functions: a capacity to localize in the nucleus when expressed alone and unknown function associated with region I.

Results

Sequencing of HPV51 L1 and L2 genes

We sequenced the L1 and L2 genes of HPV51 isolated by Matsukura and Sugase (Ma-strain) (Matsukura and Sugase, 1995) (GenBank accession numbers for L1 and L2 genes of Ma-strain are GQ487711 and GQ487712, respectively) and compared the deduced aa sequences with those of the registered HPV51 (Nuovo et al., 1988) (named Nu-strain in this study). The *aa* sequence of the Ma-strain L2 (Ma-L2) was different from that of Nu-strain L2 (Nu-L2) at 6 *aa* positions and in regions I and II (Fig. 1). The *aa* sequences of the two regions of Ma-L2 were homologous to those of the other mucosal HPVs (Fig. 1A).

The *aa* sequence of Ma-strain L1 (Ma-L1) was different at only four positions (L at *aa* 52, G at *aa* 264, S at *aa* 265, and T at *aa* 272) from those of Nu-strain L1 (Nu-L1).

Subcellular localization of the L2s

We produced codon-modified genes encoding Ma-L1, Ma-L2, and Nu-L2, using appropriate synthetic oligonucleotides. Then, six DNA fragments encoding chimeric L2s, from Ch1-L2 to Ch6-L2 (Fig. 2A), were produced by PCR-based DNA synthesis.

Fig. 2B shows immunofluorescence staining of the L2s transiently expressed alone in HEK293FT cells. HEK293 cells were transfected with the expression plasmid for L2s and fixed with paraformaldehyde 1 day later. The cells were incubated with the cross-reactive anti-L2 serum, which had been produced by immunizing mice with HPV16 L2 peptide (*aa* 11–200), and incubated with Alexa Fluor 488-conjugated anti-mouse IgG rabbit serum. L2 was detected by fluorescence microscopy. Ma-L2 and Ch3-L2 were located almost exclusively in the nucleus. Nu-L2, Ch1-L2, and Ch6-L2 were located in the cytoplasm. Ch2-L2 was located mostly in the nucleus with fluorescence somewhat weaker than that of Ma-L2, along with some cells showing fluorescence in both the nucleus and the cytoplasm. Ch4-L2 and Ch5-L2 were located in the nucleus, with some cells showing cytoplasmic fluorescence as well.

When Nu-L2 was co-expressed with Ma-L1, which has a strong nuclear localizing signal (Zhou et al., 1991), the localization of Nu-L2 was shifted to the nucleus (data not presented), suggesting that Nu-L2 made a complex with Ma-L1 and moved to the nucleus with the help of Ma-L1.

Nuclear localizing signal of HPV51 L2

Ma-L2 and Nu-L2 had two nuclear localizing signals (NSLs) at their both ends. The DNA fragments of MA-L2 corresponding regions of *aa* 5–11 (encoding RARRRDR), *aa* 95–99 (region I, encoding VIIEPIAP-TEPSIVNLVD), *aa* 179–187 (region II, encoding GHIFTSTLT), and *aa* 452–457 (encoding RKRRKR) were fused to the N-terminus of enhanced green fluorescent protein (EGFP). The resultant fusion genes were transiently expressed in HEK293FT cells and localization of EGFP was examined by fluorescent microscopy (Fig. 3). The plain EGFP was located in both of the cytoplasm and the nucleus. The EGFP with the N-terminal region or the C-terminal region was clearly accumulated in the nucleus. The localization of EGFP with region I was similar to that of plain EGFP. The EGFP with region II was located in the nucleus slightly more than in the cytoplasm in some cells, but the nuclear accumulation in those cells was much less than the EGFP with the N- or C-terminal NLS. Because the *aa* sequences of Nu-L2 N-terminal and C-terminal regions are identical with those of Ma-L2, the NSLs of Nu-L2 have the same NSL sequences. Thus, the data indicate that both terminal regions evidently contain NSLs like the L2s of HPV6b and HPV16 (Sun et al., 1995; Darshan et al., 2004), suggesting that Nu-L2 regions I and II inhibit the NLS function of Nu-L2.

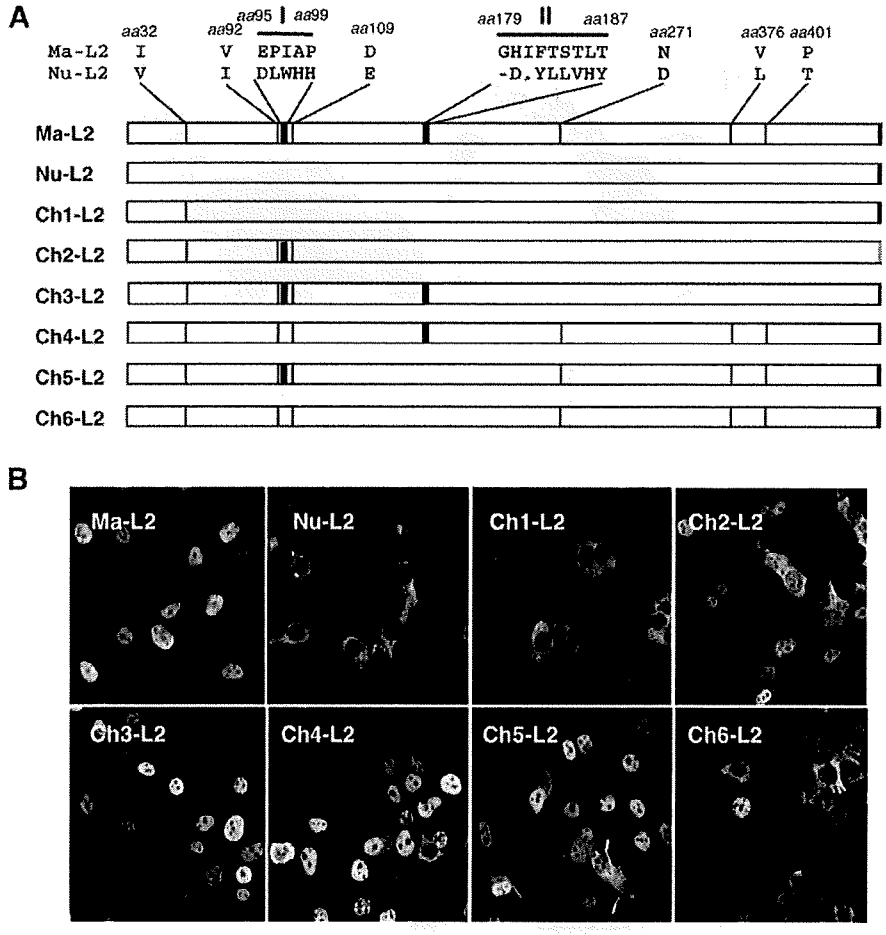


Fig. 2. Subcellular localization of the L2s. (A) Schematic representation of the L2s used in this study. (B) Subcellular localization of the L2s. 293FT cells were transfected with the expression plasmid for L2. One day later, the cells were fixed and L2 was detected with anti-L2 mouse antiserum and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen). The cells were coated with ProLong Gold anti-fade reagent (Invitrogen) and examined with a confocal microscope (FluoView1000, OLYMPUS, Tokyo, Japan).

Preparation and characterization of HPV51 pseudovirions

Since L2 is believed to play a critical role in the early process of infection (Pereira et al., 2009), we prepared and characterized HPV51 PVs containing either Ma-L2 or Nu-L2. The PVs were produced by using the codon-modified genes. HEK293FT cells were transfected with the L1- and L2-expression plasmids together with pYSEAP, a reporter plasmid expressing secretary alkaline phosphatase (SEAP). Sixty hours later the nuclear extract was centrifuged on a density-gradient as previously described (Kondo et al., 2007). A fraction containing the majority of the PVs was used as a PV preparation. The PV preparation produced with Ma-L2, Nu-L2, Ch1-L2, Ch2-L2, Ch3-L2, Ch4-L2, Ch5-L2, or Ch6-L2 was named as PV51MaL2, PV51NuL2, PV51Ch1L2, PV51Ch2L2, PV51Ch3L2, PV51Ch4L2, PV51Ch5L2, or PV51Ch6L2, respectively. For comparison, the nuclear extract from HEK293 cells transfected with the L1-expression plasmid and pYSEAP, without L2-expression plasmid, was similarly processed to produce PV51L2(−).

Transmission electron microscopy showed that PV51MaL2 and PV51NuL2 were composed of homogeneous spherical particles with a diameter of approximately 55 nm (Fig. 4A).

SDS-gel electrophoresis showed that all the PV preparations, except for PV51L2(−), contained both L1 and L2 (Fig. 4B). The

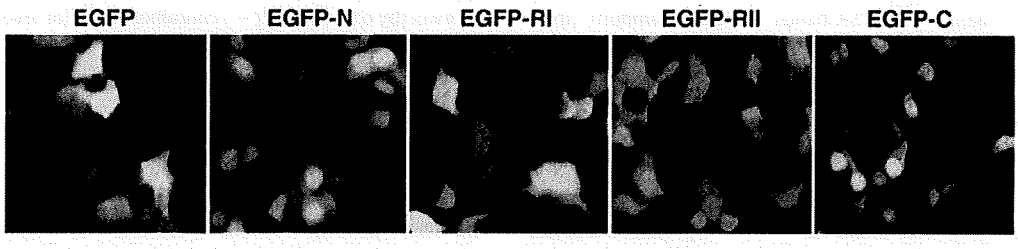
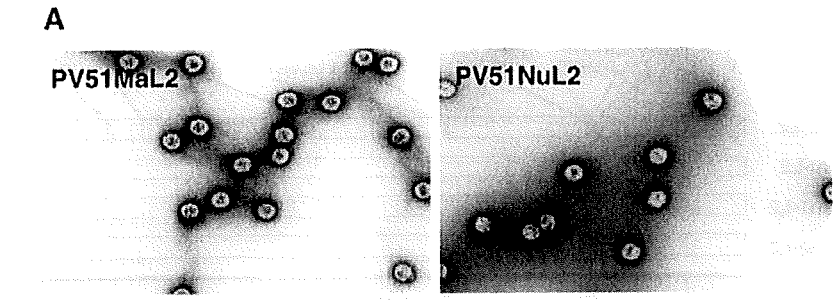
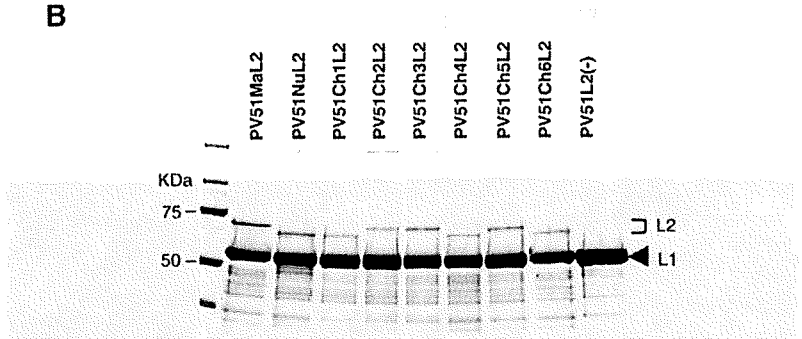


Fig. 3. Subcellular localization of the enhanced green fluorescent protein (EGFP) fused with nuclear localizing signal of Ma-L2. 293FT cells were transfected with the expression plasmid for EGFP or EGFP fused with N-terminal region (*aa* 5–11) (EGFP-N), region I (*aa* 92–109) (EGFP-RI), region II (*aa* 179–187) (EGFP-RII), and C-terminal region (*aa* 452–457) (EGFP-C). One day later, the cells were fixed and examined with a confocal microscope (FluoView1000, OLYMPUS, Tokyo, Japan).





C

	DNase resistant SEAP DNA (ng/1 μgL1protein)
PV51MaL2	0.6
PV51NuL2	0.9
PV51Ch1L2	1.4
PV51Ch2L2	0.8
PV51Ch3L2	0.7
PV51Ch4L2	0.9
PV51Ch5L2	1.5
PV51Ch6L2	1.2
PV51L2(-)	1.5

Fig. 4. Characterization of HPV51 pseudovirion preparations. (A) Electron micrograph of PV51MaL2 and PV51NuL2. The pseudovirion preparation was allowed to settle on carbon-coated copper grids and negatively stained with 4% uranylacetate. The grids were examined in a HITACHI model H-7600 transmission electron microscope and photographed at an instrumental magnification of ×200,000. Bar = 50 nm. (B) SDS-gel electrophoresis of the pseudovirion preparations. The pseudovirion preparation was mixed with an equal volume of SDS sample buffer and boiled. Then the sample was electrophoresed on 10% SDS-polyacrylamide gel. Protein was stained with SYPRO Ruby Protein Gel Stain (Life Technologies Corp.) and detected with a Typhoon 9410 image analyzer (GE Healthcare Life Sciences). (C) Level of the reporter plasmid packaged in the pseudovirion. The mixture of the pseudovirion preparation and DNase-I was incubated at 37 °C for 1 hour. DNase-resistant DNA was extracted and quantified by real-time PCR with primers complementary to the reporter plasmid, pYSEAP.

aliquots of the preparation were electrophoresed on an SDS-polyacrylamide gel and stained with SYPRO Ruby Protein Gel Stain (Life Technologies Corp.). Protein in the gel was detected with a Typhoon 9410 image analyzer (GE Healthcare Life Sciences). The L1-contents in the PV preparations, which were estimated by comparison of L1-bands with BSA-bands of known amount (from 0.01 to 0.1 μg per lane) on an SDS-gel (data not shown), ranged from 0.4 to 0.8 μg/μl. The PV preparation containing approximately 1 μg of L1 was electrophoresed and the proteins in the gel were stained similarly. PV51MaL2 contained slightly more L2 than the other PVs. Nu-L2, Ch2-L2, Ch3-L2, and Ch5-L2 migrated faster than the other L2s, indicating that region I was associated with the faster migration.

The PV preparations were found to contain the packaged reporter plasmid at a similar level (Fig. 4C). The PV preparations containing 2 to 4 μg of L1 were digested with DNase-I extensively and the level of the DNase-resistant pYSEAP was measured by a real-time PCR method. The numbers of reporter copies and the particles were calculated from the DNA and L1 contents, respectively. The PV preparations were estimated to contain approximately 0.3 to 1 genome copies per 100 particles; that is, the complete PVs constituted approximately 0.3% to 1% of the particles in a preparation. It is noteworthy that PV51L2(−) contained a similar level of the packaged reporter, indicating that the presence of L2 did not affect the packaging efficiency.

Expression of the reporter in HEK293FT cells inoculated with HPV51 pseudovirions

Fig. 5 shows the levels of reporter expression in HEK293FT cells inoculated with the PVs. The 293FT cells (10^4 cells) were inoculated with the PV preparation containing approximately 5×10^4 genome copies and incubated at 37 °C for 3 days. Then, the SEAP activity of the culture medium was measured. The cells inoculated with PV51L2(−)

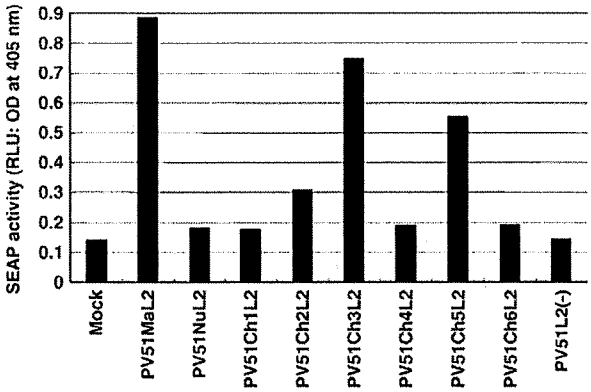


Fig. 5. Expression of secretary alkaline phosphatase (SEAP) in 293FT cells inoculated with the pseudovirion preparations. HEK293 cells inoculated with the pseudovirion preparation were incubated for 3 days and SEAP activity of the culture medium was measured by the colorimetric SEAP assay. Mock: SEAP activity of the culture medium of the cells not inoculated with the pseudovirion. RLU: relative light unit.

did not express the reporter, indicating that L2 is required for the reporter expression. The cells inoculated with PV51MaL2, PV51Ch3L2, and PV51Ch5L2 expressed the reporter efficiently and those with PV51Ch2L2 expressed less efficiently. The cells inoculated with PV51NuL2, PV51Ch1L2, PV51Ch4L2, and PV51Ch6L2 did not express the reporter. Evidently, L2s in PV51NuL2, PV51Ch1L2, PV51Ch4L2, and PV51Ch6L2 lacked the function required for the reporter expression.

Discussion

This study showed that the prototype HPV51 (Nuovo et al., 1988) is likely defective because the PV containing its L2 lacks infectivity (Fig. 5). Regions I and II of the prototype L2 were markedly different from those of the new isolate (Matsukura and Sugase, 1995), which were homologous to those of the other mucosal HPVs (Figs. 1A and B). The prototype may have been cloned from defective DNA that was integrated in the chromosome or maintained as an episome in the cervical condyloma.

Ma-L2 expressed alone in HEK293FT cells was located exclusively in the nucleus (Fig. 2B), perhaps by using its NLSs at the both ends as previously shown for L2s of HPV6b and 16 (Sun et al., 1995; Darshan et al., 2004). By contrast, Nu-L2 expressed in HEK293FT cells was located exclusively in the cytoplasm (Fig. 2B), despite the presence of NLS. Regions I and II of Nu-L2 probably affect the conformation of Nu-L2 and the resultant change may inhibit the activity of NSLs. The NLS function of chimeric L2s, having either region I or region II of Nu-L2 (Ch2-L2, Ch4-L2, and Ch5-L2), may have been partially reduced and that of chimeric L2s, having both regions (Ch1-L2 and Ch6-L2), markedly reduced.

Nu-L2 and three chimeric L2s, Ch1-L2, Ch4-L2, and Ch6-L2, migrated faster than the other L2s on the SDS-gel electrophoresis (Fig. 4B). Because only these L2s contain region I of Nu-L2, the faster migration is probably associated with the conformational modification induced by Nu-L2 region I.

Nu-L2 was translocated to the nucleus in the cells expressing Ma-L1, suggesting that Nu-L2 binds to L1 and makes a complex, as described previously for HPV11 L1 and L2 (Finnen et al., 2003), and the complex moves to the nucleus by using the NLS of L1. The L1-mediated translocation seems to make L2s with the reduced NSL activities possible to assemble into the particles in the nucleus.

PV51MaL2 and PV51NuL2 contained spherical particles (Fig. 4A) composed of L1 and L2 (Fig. 4B). PV51MaL2, PV51NuL2, PV51Ch1L2, PV51Ch2L2, PV51Ch3L2, PV51Ch4L2, PV51Ch5L2, and PV51Ch6L2 contained L1 and L2 at a comparable ratio and the DNase-resistant (very likely packaged) reporter plasmid at similar levels (Fig. 4C). However, the reporter was expressed in HEK293FT cells inoculated with PV51MaL2, PV51Ch2L2, PV51Ch3L2, and PV51Ch5L2 but not with Pv51NuL2, Pv51Ch1L2, or Pv51Ch4L2, and Pv51Ch6L2 (Fig. 5). Thus, the PVs having Ma-L2 region I induced the expression of the reporter. These L2s are capable of localizing in the nucleus and migrate slower than the other L2s in the SDS-gel electrophoresis. As reported previously for L2 of HPV16 (Day et al., 2004), probably L2 binds to the packaged DNA and takes it to the nucleus. Then, an unknown second function, which may be associated with L2 conformation maintained by the *aa* sequence of Ma-L2 region I, helps the expression of the reporter plasmid. Ch4-L2, which was located in the nucleus but migrated faster, probably lacks the second function.

The reporter plasmid was packaged into PV51L2(−) similarly, indicating that L2 is not involved in the packaging (Fig. 4C) as reported by Buck et al. (2005). The particles containing the reporter plasmid were approximately 0.3% to 1% of the particles in the PV preparations. The extremely low packaging efficiency suggests that the packaging occurs by chance. For the survival of HPV51 in humans, HPV genomic DNA must have an unidentified mechanism, such as a packaging signal, to induce efficient packaging.

In summary, this study clearly showed that although HPV51 L2 is not involved in the packaging of the reporter into the L1/L2-VLP in the process of pseudovirion production, the expression of the packaged reporter requires the functions of L2: its nuclear localizing capacity and unknown function associated with *aa* 95–99.

Materials and methods

Cell

HEK293FT cells, a cell line expressing a high level of SV40 T-antigen, was purchased from Life Technologies Corp. (Carlsbad, CA, USA). The cells were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Life Technologies Corp.), 1% GlutaMax-I (Life Technologies Corp.), and Geneticin (500 μg/ml; Life Technologies Corp.).

Expression plasmids

The L1 and L2 genes of the cloned Ma-strain HPV51 DNA (a gift from Dr. Toshihiko Matsukura) (Matsukura and Sugase, 1995) were sequenced with an ABI 3130xl sequencer (Life Technologies Corp.). The *aa* sequences of L1 and L2 of Matsukura strain were deduced from the nucleotide sequences and those of Nuovo strain were deduced from the nucleotide sequences in the database (GenBank M62877). The nucleotide sequence of the codon-modified gene encoding the L1 or the L2 was designed with the following codons, which are used most frequently in human mRNAs; Ala: GCC; Cys: TGC; Asp: GAC; Glu; GAG; Phe: TTC; Gly: GGC; His: CAC; Ile: ATC; Lys: AAG; Leu: CTG; Asn: AAC; Pro: CCC; Gln: CAG; Arg; AGG; Ser: AGC; Thr: ACC; Val: GTG; Trp: TGG; Tyr: TAC. The codon-modified genes were produced by PCR with sense and antisense synthetic oligonucleotides that had an annealing region (approximately 20 nucleotides) at its 3′ end. HPV16L1 gene inserted between *Not*I and *Hin*dIII sites in p16L1h (gifts from J. T. Schiller), a plasmid expressing the codon-modified HPV16 L1, was replaced with the codon-modified L2 genes encoding Ma-L1, Ma-L2, and Nu-L2 to produce pMa-L1, pMa-L2, and pNu-L2, respectively.

Three regions of the codon-modified Nu-L2 gene, nt1-96, nt1-327, and nt1-561 (A at the first ATG of Nu-L2 gene was numbered as nt1) were replaced with corresponding regions of codon-modified Ma-L2 genes by using PCR to produce Ch1-L2, Ch2-L2, and Ch3-L2 genes, respectively. The regions of the codon-modified Ma-L2 gene, nucleotide (*nt*) 285 to 296 and *nt* 536 to 560 were replaced with corresponding regions of codon-modified Nu-L2 gene by using PCR to produce Ch4-L2 and Ch5-L2 genes, respectively. The region of Ch4-L2

gene, nt1 to nt456, was ligated with the region of Ch5-L2 gene, nt457 to nt1410, at the *PmlI* site to produce Ch6-L2 gene. These genes were inserted similarly to p16L1h to produce pCh1-L2, pCh2-L2, pCh3-L2, pCh4-L2, pCh5-L2, and pCh6-L2, respectively.

Expression plasmids for EGFP fused with L2 NSLs were produced by insertion of the annealed synthetic oligonucleotides encoding MVAT plus none, *aa* 5–11, *aa* 92–109, *aa* 179–187, and *aa* 452–457 of Ma-L2 at the multicloning site of EGFP gene (pCMS-EGFP; Clontech Inc. Mountain View, CA). The resultant fusion EGFP contained additional *aa* sequences at its N-terminus.

The plasmid expressing secretary alkaline phosphatase from SV40 early promoter, pYSEAP, was a gift from J. T. Schiller.

The nucleotide sequences of the all the plasmids constructed in this study were verified by sequencing.

Fluorescence microscopy

HEK293FT cells in an 8-well chamber plate (Nunc, Rochester, NY) were transfected with 0.2 μg of the expression plasmid for L2 by using Optifect transfection reagent (Invitrogen). One day later, the cells were fixed with 4% paraformaldehyde in PBS and incubated with 0.5% Triton X-100 in PBS. The cells were reacted with anti-L2 serum obtained by immunizing mice with HPV16L2 peptide (the region of *aa* 11–200), which cross-reacts with L2s of various mucosal HPVs, followed by Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen). The cells were coated with ProLong Gold anti-fade reagent (Invitrogen) and examined with a confocal microscope (FluoView1000, OLYMPUS, Tokyo, Japan).

HEK293FT cells in an 8-well chamber plate (Nunc, Rochester, NY) were transfected with 0.2 μg of the expression plasmid for EGFP fused with Ma-L2 NSL by using Optifect transfection reagent (Invitrogen). One day later, the cells were fixed with 4% paraformaldehyde in PBS, coated with ProLong Gold anti-fade reagent (Invitrogen), and imaged in a BZ-8000 fluorescence microscope (Keyence, OSAKA, Japan).

Production of pseudovirions

HEK293FT cells (Life Technologies Corp.), which had been seeded in a 10-cm culture dish ($1X10^7$ cells) at 16 hours before the transfection, were transfected with a mixture of an L1-plasmid (13.5 μg), an L2-plasmid (3 μg), and pYSEAP (13.5 μg) by using Fugene HD (Rosch Diagnostics GmbH, Mannheim, Germany). Sixty hours later, the cells were harvested with trypsin. The cells were suspended in 0.5 ml of lysis buffer (PBS containing 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.35% Brij58 [Sigma-Aldrich Inc., St. Louis, MO], 0.1% Benzonase [Sigma-Aldrich Inc.], 0.1% Plasmid Safe ATP dependent-DNase [EPICENTRE Corp. Madison, WI]) and incubated for 30 hours at 37 °C with slow rotation. The lysate was cooled on ice for 5 minutes, mixed with 5 M NaCl solutions to adjust the concentration of NaCl to 0.85 M, and further kept on ice for 10 minutes. Then, the lysate was centrifuged at 5000g for 10 minutes at 4 °C. The supernatant was layered on an Optiprep gradient (from top to bottom, 27%, 33%, and39% in PBS containing 1 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.8 M NaCl) and centrifuged at 50,000 rpm for 3.5 hours at 16 °C in an SW55Ti rotor (Beckman Coulter Inc. Fullerton, CA). Fractions (300 μl each) were obtained by puncturing the bottom. Aliquots (5 μl per fraction) were analyzed by SDS-gel electrophoresis. The fraction that contained L1 most abundantly was used as a pseudovirion (PV) preparation.

Electron microscopy

The particles in a PV preparation were allowed to settle on carbon-coated copper grids and stained with 4% uranylacetate. The grids were examined in a HITACHI model H-7600 transmission electron microscope and photographed at an instrumental magnification of ×200,000.

Quantification of L1 protein

A PV preparation (0.1 μl) or standard protein solution consisting of the known amount (~0.1–1 μg/μl) of bovine serum albumin (BSA) was mixed with an equal volume of SDS sample buffer and boiled. These samples were electrophoresed on 10% SDS-polyacrylamide gel. Protein was stained with SYPRO Ruby Protein Gel Stain (Life Technologies Corp.) and detected with a Typhoon 9410 image analyzer (GE Healthcare Life Sciences). The amount of L1 protein in the PV preparation was estimated from comparing the images obtained with a Typhoon 9410 imaging analyzer and Image Quant TL software (GE Healthcare, Chalfont St. Giles, UK).

Quantification of DNase-resistant reporter DNA

Five microliters of a PV preparation was mixed with 195 μl of PBS containing $MgCl_2$ (10 mM) and DNase-I (70 U). After incubating at 37 °C for 1 hour, DNA was extracted by using QIAamp DNA Blood kit (Qiagen GmbH, Hilden, Germany). pYSEAP DNA in the sample was quantified by real-time PCR using forward primer (AGAACCCG-GACTTCTGGAAC) and reverse primer (GGCAGCTGTCACCGTAGACA).

Quantification of SEAP activity

A PV preparation was inoculated to HEK293FT cells (2×10^4), which had been seeded 1 day prior to the inoculation in 96-well flat-bottom tissue culture-treated plates (Corning Costar Corp., New York, NY). The cells were incubated for 3 days and SEAP activity of the culture medium was measured by the colorimetric SEAP assay (NCI home page: http://home.ccr.cancer.gov/lco/colorimetricseap.htm).

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Identification of nucleolin as a protein that binds to human papillomavirus type 16 DNA

Hidetaka Sato [a,b], Rika Kusumoto-Matsuo [a], Yoshiyuki Ishii [a], Seiichiro Mori [a], Tomomi Nakahara [a], Fumiko Shinkai-Ouchi [c], Kei Kawana [b], Tomoyuki Fujii [b], Yuji Taketani [b], Tadahito Kanda [a], Iwao Kukimoto [a,*]

[a] Pathogen Genomics Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[b] Department of Obstetrics and Gynecology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT

Transcription, replication, and segregation of human papillomaviruses (HPVs) are regulated by various host factors, but our understanding of host proteins that bind to the HPV genome is limited. Here we report the results of a search of cellular proteins that can associate with specific genomic regions of HPV type 16 (HPV16). We found that human nucleolin, an abundant nucleolar protein, was preferentially captured *in vitro* by an HPV16 genomic fragment from nucleotide positions (nt) 531–780. Electrophoretic mobility shift assays with a bacterially expressed nucleolin revealed that nucleolin bound to an HPV16 genomic region between nt 604 and 614 in a sequence-dependent manner. Chromatin immunoprecipitation analysis showed that both exogenous and endogenous nucleolin bound to a plasmid containing the HPV16 genomic region in HeLa cells. Furthermore, nucleolin associated with the HPV16 genome stably maintained in HPV16-infected W12 cells, suggesting that the nucleolin binding may be involved in the dynamics of the HPV genome in cells.

Introduction

Human papillomaviruses (HPVs), which are recognized as the causative agents of cervical cancer, have circular double-stranded DNA genomes with sizes close to 8 kbp [1]. HPV infects basal cells in the epidermis and its genome is maintained as episomes, whereas the viral genome amplification occurs in upper differentiating epithelium [2]. Due to the limited coding capacity of its small genome, HPV relies heavily on the function of host cell proteins for viral transcription, replication and segregation [3,4]. The transcription of the HPV genome is driven by two major promoters: in HPV type 16 (HPV16) genome, the early promoter P_{97} directs early gene transcription, while the late promoter P_{670} induces capsid expression. After initial unwinding of the replication origin by the coordinated action of the HPV E1 and E2 proteins, the HPV DNA replication progresses with the use of cellular replication proteins. For viral genome maintenance, the HPV genome is passively segregated by being tethered to host chromosomes, then passed into nuclei of daughter cells. Because of a lack of cell culture systems for efficient HPV propagation, molecular mechanisms of these processes are not fully understood.

Nucleolin is an abundant, ubiquitously expressed protein that is found in the nucleolus, the nucleoplasm, and on the cell surface, and is involved in regulation of ribosomal DNA (rDNA) transcription and the maturation of pre-ribosomal RNA [5]. In addition, nucleolin exerts several nuclear functions related to the transcription of several genes by the RNA polymerase II [6–8], genotoxic stress response [9], and chromosome congression in mitosis [10].

To gain new insights into how the dynamics of the HPV genome are regulated by host factors, we searched for cellular proteins that can bind to the two promoter regions of HPV16 using an unbiased proteomic approach. We describe the identification and characterization of nucleolin as an HPV16 genome-binding protein that may play a role in regulation of the HPV life cycle.

Materials and methods

Isolation of HPV16 genome-binding protein and its identification by peptide mass fingerprinting. Three HPV16 DNA fragments, I (nt 7791–120, 234 bp), II (nt 131–360, 230 bp), and III (nt 531–780, 250 bp), were generated by PCR using following primers: I forward, 5′biotin—TAC ATG AAC TGT GTA AAG GTT AGT CA-3′; I reverse, 5′-TGT GGG TCC TGA AAC ATT GCA GTT CTC TTT-3′; II forward, 5′biotin—AGA AAG TTA CCA CAG TTA TGC ACA GA-3′; II reverse, 5′-GTT CCA TAC AAA CTA TAA CAA TAA TGT CTA-3′; III forward, 5′biotin—CAA GAA CAC GTA GAG AAA CCC AGC TG-3′; III reverse, 5′-GTG TGT

* Corresponding author. Address: Pathogen Genomics Center, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashi-murayama, Tokyo 208-0011, Japan. Fax: +81 42 567 5632.
E-mail address: ikuki@nih.go.jp (I. Kukimoto).

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GCT TTG TAC GCA CAA CCG-3′. The biotinylated PCR products were purified using the Wizard SV gel and PCR clean-up system (Promega) and coupled to Dynabeads M-280 streptavidin (Dynal, Norway) in a buffer consisting of 10 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl. The HeLa nuclear extract was prepared by Dignam's procedure and incubated with the HPV16 DNA-coupled magnetic beads at 4 °C overnight. The beads were then washed three times in a wash buffer (10 mM Hepes, pH 7.9, 200 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and 10% glycerol). The bound proteins were released from the beads in SDS-sample buffer by boiling for 5 min and fractionated in a 4–20% gradient SDS–polyacrylamide gel (Daiichi Pure Chemicals, Japan), followed by silver staining. The 95-kDa protein band bound to fragment III was excised from the gel and subjected to in-gel trypsin digestion. The resultant peptide mixtures were analyzed by MALDI-QIT-TOF MS (AXIMA-QIT, Shimazu Biotech, Japan). Mascot software (Matrix Science) was used for protein identification.

Electrophoretic mobility shift assay (EMSA). The full-length cDNA of human nucleolin was amplified by RT-PCR from mRNA of HeLa cells. The resultant cDNA sequence completely matched the nucleolin sequence in the GenBank (NM 005381). To generate an expression plasmid for glutathione *S*-transferase (GST) fused nucleolin, the cDNA fragment encoding nucleolin amino acid (aa) from 289 to 710 was amplified by PCR with the full-length nucleolin cDNA as a template, and cloned into pGEX-2TK (GE Healthcare). GST–nucleolin and GST were expressed in *Escherichia coli* and purified using a GSTrap HT column and an AKTAprime (GE Healthcare). The EMSA was performed as described previously [11]. The DNA/protein complex was separated on a 5% polyacrylamide gel and visualized by autoradiography on X-ray films.

Chromatin immunoprecipitation (ChIP) assay. An expression plasmid for N-terminally FLAG-tagged nucleolin (FLAG-nucleolin) was constructed by cloning the full-length cDNA of nucleolin into p3xFLAG-CMV10 (Sigma). The ChIP assay was performed as described previously [11] with some modifications. Briefly, HeLa cells were transfected with pGL3-P_{670} or pGL3-Basic (Promega) together with the FLAG-nucleolin expression plasmid or p3xFLAG-CMV10 using FuGENE6 (Roche). At 48 h after the transfection, the cells were fixed with 1% formaldehyde at 37 °C for 5 min, lysed and sonicated using a Bioruptor (Cosmobio, Japan). The sonicated extract was immunoprecipitated with specific antibodies that had been coupled to Dynabeads M-280 sheep anti-mouse IgG (Dynal). Antibodies used were anti-FLAG M2 (Sigma), anti-nucleolin (MS-3, Santa Cruz, or 4E2, Abcam), or control mouse IgG (Santa Cruz). After washing the beads, the immunoprecipitated DNA/protein complexes were eluted, and reverse cross-linked. DNA was purified and subjected to PCR for HPV16 DNA (from nt 501 to 670), 18S rDNA, or pGL3-Basic. PCR primers were as follows: HPV16 forward, 5′-CCG GTC GAT GTA TGT CTT GTT GCA GAT CAT-3′; HPV16 reverse, 5′-CAT CCT CCT CCT CTG AGC TGT CAT TTA ATT-3′; 18S rDNA forward, 5′-GCC TGG ATA CCG CAG CTA GGA ATA ATG G-3′; 18S rDNA reverse, 5′-TTG ATT AAT GAA AAC ATT CTT GGC AAA TG-3′; pGL3-Basic forward, 5′-AGA CCC ACG CTC ACC GGC TCC AGA-3′; pGL3-Basic reverse, 5′-ACG AGC GTG ACA CCA CGA TGC CTG T-3′. The amounts of the immunoprecipitated DNA were quantified by real-time PCR analysis using a LightCycler 480 (Roche) with the LightCycler 480 SYBR Green I Master reagent (Roche). W12 cells were cultured in an undifferentiated state as described [12], and the ChIP assay was performed without plasmid transfection.

Generation of nucleolin-knockdown cells and Western blotting. The stable nucleolin-knockdown cell line derived from HeLa cells was established by transfection of an expression plasmid for small hairpin RNA (shRNA) against nucleolin and subsequent selection of cells in the presence of 10 μg/ml puromycin. The shRNA-expression plasmid was constructed by cloning the shRNA target sequence for nucleolin (5′-GGA AGA CGG TGA AAT TGA T-3′) [13] into pBAsi-hU6 (Takara, Japan). For Western blot analysis, cell extracts were prepared by boiling cells in SDS-sample buffer. Protein samples were separated on a 12% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany), and probed with a specified primary antibody and a peroxidase-conjugated secondary antibody. Antibodies used were anti-nucleolin (MS-3, Santa Cruz, or 3G4B20, Active Motif), anti-PCNA (PC10, Santa Cruz), and anti-FLAG M2. Specific proteins were visualized using an ECL Western blot detection system (GE Healthcare).

Results

Identification of nucleolin as a protein binding to an HPV16 genomic fragment

Two regions in the HPV16 genome were chosen as targets to screen for cellular proteins that might potentially regulate transcription and/or replication of HPV (I and III in Fig. 1A). Fragment I (nt 7791–120) contains the regulatory sequence for the HPV16 early promoter P_{97} and the replication origin, whereas fragment III (nt 531–780) includes the late promoter P_{670}. To find specific binding proteins for these fragments, fragment II (nt 131–360) was used as a control for comparison, because no particular binding proteins were assigned to this region. These DNA fragments were generated by PCR so as to have 5′-biotin-labeled ends, coupled to streptavidin-conjugated magnetic beads and then incubated in a nuclear extract prepared from HeLa cells. After washing the beads, bound proteins were released and separated by SDS–PAGE, followed by silver staining. Among many protein bands detected, fragment III selectively bound to a 95-kDa protein (Fig. 1B), while no specific proteins were detected for fragment I. To identify the 95-kDa protein, the band was excised from the gel, digested with trypsin, then subjected to peptide mass fingerprinting. The list of observed mass fingerprints significantly fitted the predicted mass fingerprint of human nucleolin (p value = 0.0094). Western blot analysis with an anti-nucleolin antibody verified an enrichment of nucleolin in the bound fraction of fragment III (Fig. 1C).

In vitro binding of nucleolin to the HPV16 DNA

An electrophoretic mobility shift assay (EMSA) revealed that a recombinant nucleolin bound to a site in the HPV16 genome from nt 591 to 620. Human nucleolin from amino acid 289 to 710, which contains four RNA-binding domains and a C-terminal glycine/arginine-rich region (Fig. 2A), was expressed as a fusion protein with GST (GST–nucleolin) in bacteria and affinity purified (Fig. 2B). This truncated nucleolin has been shown to exhibit DNA-binding activity [6]. GST–nucleolin was examined in EMSA for its capability to form a complex with overlapping DNA probes having the HPV16 sequences from nt 531 to 660. Among the HPV16 probes tested, probe f generated a shifted band with GST–nucleolin (Fig. 2C).

Nucleolin has been previously reported to bind to a different location in the HPV18 genome *in vitro*, and the binding site has been assigned to the sequence 5′-TTGCTTGCATAA-3′ (nt 7642–7653 in the HPV18 genome) [14]. Similarity between the HPV18 sequence and probe f was explored, and the same sequence motif, 5′-TTGCXXXCAXA-3′, was found in the two sequences (Fig. 2D, upper panel). To test whether this sequence motif was recognized by nucleolin, base substitutions that have been shown to abolish the nucleolin binding to the HPV18 site were introduced into probe f. The mutations completely disrupted the GST–nucleolin binding to the probe (Fig. 2D), demonstrating that nucleolin bound to this motif in probe f in a sequence-dependent manner.

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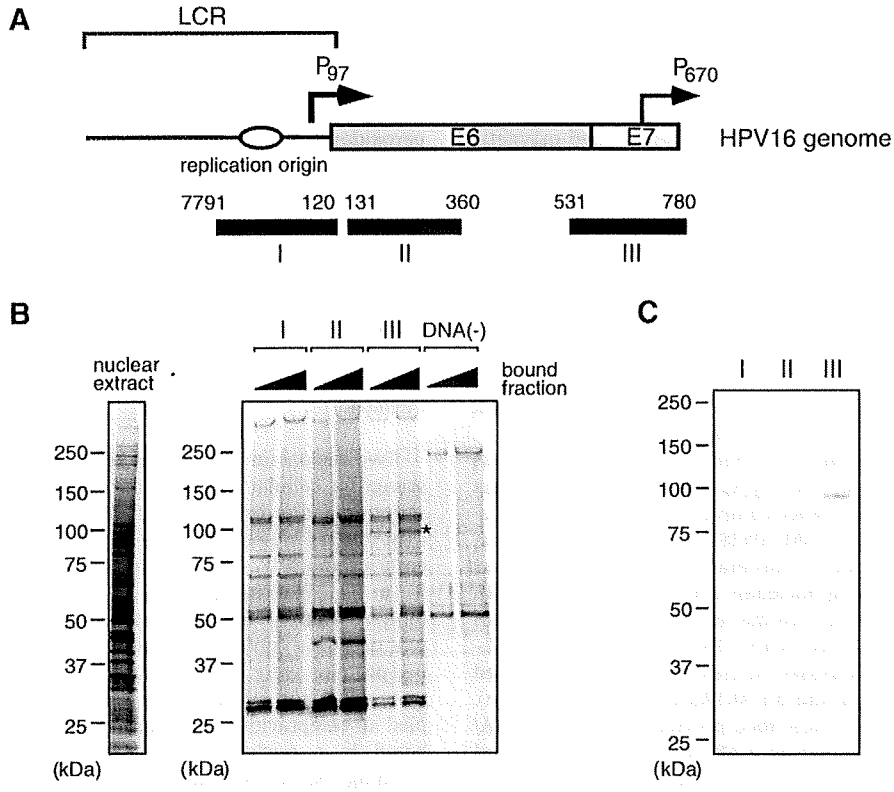

Fig. 1. Isolation of cellular proteins bound to HPV16 genomic fragments. (A) Schematic for locations of three HPV16 DNA fragments (I, II, and III) used to screen for binding proteins to HPV16 DNA. Numbers above the fragments indicate nucleotide positions in the HPV16 genome. The positions of the early promoter (P_{97}), the late promoter (P_{670}), the long control region (LCR), and the replication origin are presented. (B) The nuclear extract of HeLa cells was incubated with magnetic beads that were coupled with the HPV16 DNA fragments or with the beads alone. The bound fractions were recovered and resolved by SDS–PAGE, followed by silver staining. A 95-kDa band bound to fragment III is indicated by an asterisk. (C) Western blot analysis of the bound fractions using anti-nucleolin antibody (Santa Cruz).

GST–nucleolin bound to an HPV18 probe having the nucleolin-binding motif (Fig. 2D), which confirmed the integrity of our GST–nucleolin preparation. Additionally, several base substitutions were introduced into the motif in probe f (Fig. 2E, upper panel). All mutated probes gave rise to a band shift more efficiently than the original probe f (Fig. 2E), indicating that nucleolin recognizes this motif but it is not an optimal sequence for nucleolin binding.

Binding of nucleolin to the HPV16 DNA in cells

Chromatin immunoprecipitation (ChIP) analysis demonstrated that exogenous nucleolin bound to the HPV16 DNA in cells. An expression plasmid for FLAG-tagged nucleolin (FLAG-nucleolin) (Fig. 3A) was constructed and used for ChIP analysis. HeLa cells were transfected with the HPV16 reporter plasmid pGL3-P_{670}, which contains the HPV16 genomic region from nt 7003 to 868 [11], with or without the FLAG-nucleolin expression plasmid. In the presence of FLAG-nucleolin, an anti-FLAG antibody precipitated the HPV16 DNA fragment containing from nt 501 to 670 compared to the control IgG precipitate (Fig. 3B). Without FLAG-nucleolin expression, the anti-FLAG antibody did not precipitate the HPV16 DNA. The anti-FLAG antibody recovered an endogenous target of nucleolin, 18S rDNA, which indicated that exogenous FLAG-nucleolin behaved as endogenous one. The backbone plasmid lacking the HPV16 sequence was not precipitated with the anti-FLAG antibody in the presence of FLAG-nucleolin (Fig. 3C), demonstrating that the HPV16 sequence was responsible for the FLAG-nucleolin binding in cells. Similar results were obtained with human primary foreskin keratinocytes (data not shown), suggesting that the nucleolin binding to the HPV16 DNA is not specific to cancer cells.

Endogenous nucleolin also associated with the HPV16 DNA in cells. When the ChIP assay was performed in HeLa cells transfected with pGL3-P_{670} alone, the precipitate with an anti-nucleolin antibody showed an enrichment of the HPV16 DNA compared to basal level DNA obtained with a control antibody (Fig. 3D). To further examine binding properties of nucleolin to the HPV16 DNA in cells, a stable HeLa cell line expressing a reduced level of nucleolin was generated using an shRNA-mediated knockdown technique. Western blot analysis showed that the nucleolin level was reduced by one-third in the knockdown cells relative to parental cells (Fig. 3E). The ChIP assay revealed that the binding of nucleolin to the transfected HPV16 DNA was weakened in the knockdown cells compared to wild-type cells, and a similar reduction of nucleolin binding to rDNA loci was observed (Fig. 3F), suggesting that nucleolin's affinity for the HPV16 DNA is comparable to that for endogenous rDNA loci.

The ChIP assay was further extended to another human cell line, W12 cells, established from a cervical intraepithelial lesion and shown to maintain up to 1000 copies of the complete HPV16 genome as episomes in cell culture [12,15]. The immunoprecipitation with the anti-nucleolin antibody from the cross-linked chromatin of W12 cells enriched the HPV16 genomic DNA two to threefold compared to the control IgG precipitate, and a similar enrichment was observed with rDNA (Fig. 4). The results suggest that endogenous nucleolin is inherently bound to the HPV16 genome in the HPV16-infected cells.

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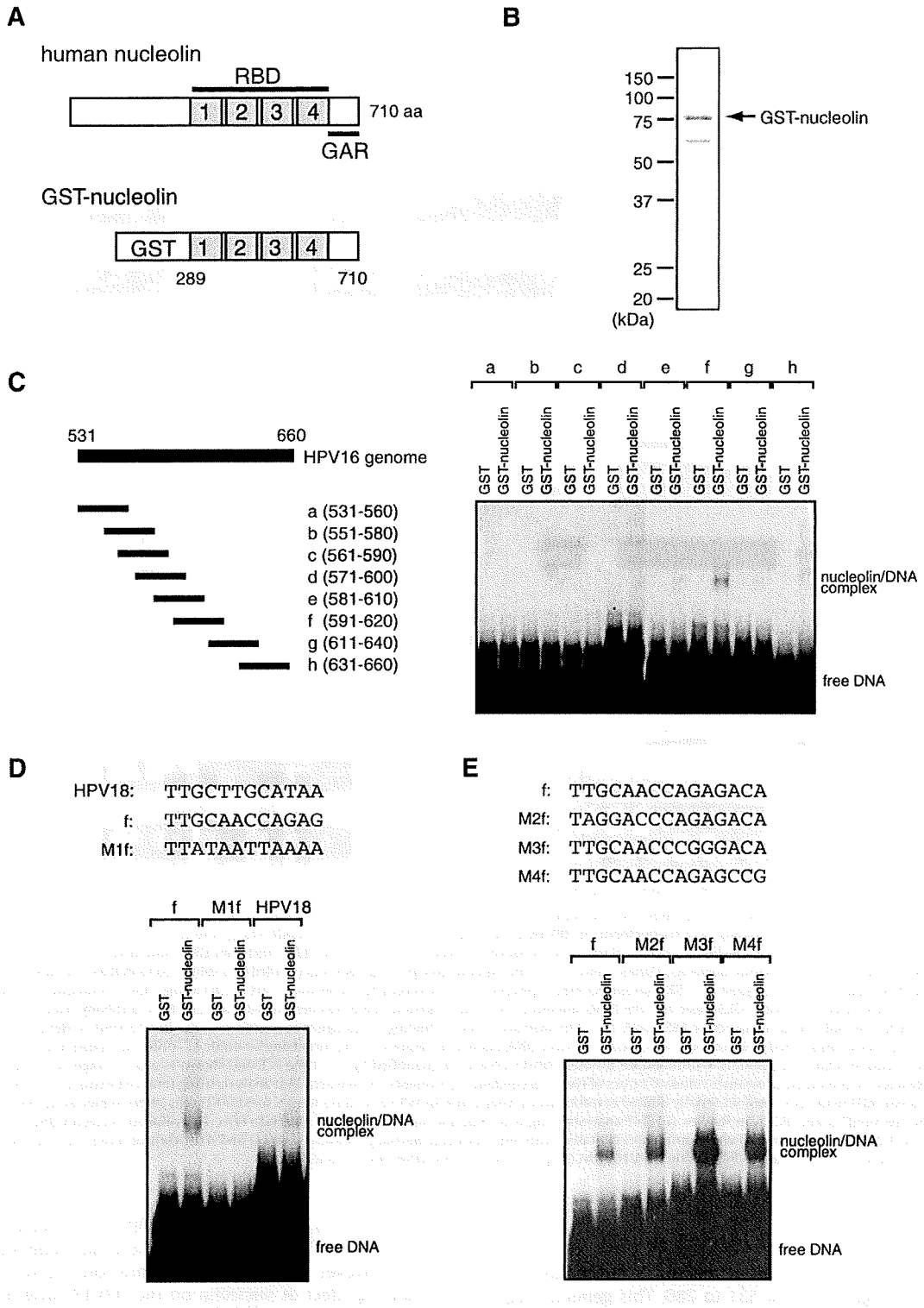

Fig. 2. *In vitro* binding of nucleolin to HPV16 DNA. (A) Schematic representation of human nucleolin and recombinant GST–nucleolin. Four RNA-binding domains (RBD1–4) and the glycine/arginine-rich region (GAR) are indicated. (B) SDS–PAGE analysis of GST–nucleolin with GelCode stain (Pierce). (C) EMSA showing a complex formation between GST–nucleolin and [^{32}P]-labeled oligonucleotide probes having the HPV16 genome sequence from nt 531 to 660. The genomic locations of the probes are presented on the left. (D) EMSA using mutated probe f to examine a sequence-specific binding of GST–nucleolin. Sequence alignment among the HPV18 nucleolin-binding sequence, probe f, and mutated probe f (M1f) is shown above. (E) Mutational analyses of probe f by EMSA. Base substitutions introduced into probe f are shown above.

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