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Human Papillomavirus Oncoprotein E6 Binds to the C-Terminal Region of Human Minichromosome Maintenance 7 Protein

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Oncoprotein E6 of the human papillomavirus (HPV) associated with cervical cancer (HPV-16 and -18) degrades tumor suppressor protein p53, but seems to have p53-independent transforming functions. We searched for other cellular targets for the N-terminal region of HPV-16 E6 using a yeast two-hybrid system. The E6 was found to bind to the C-terminal region of a human minichromosome maintenance 7 (hMCM7) protein, which is a component of replication licensing factors. The full-length hMCM7 translated *in vitro* was capable of binding to bacterially expressed E6. In yeast cells the E6s of the cancer-associated HPVs (HPV-16, -18, and -58) bound to hMCM7 more strongly than those of the HPVs associated with a benign tumor (HPV-6 and -11). Binding of E6 with hMCM7 may cause chromosomal abnormalities found in the human cells expressing E6s of oncogenic HPVs.

Among human papillomaviruses (HPVs) associated with the lesions in the anogenital tract, types predominantly found in cervical cancer and high-grade intraepithelial neoplasias are referred to as "high-risk"(HPV-16 and -18) and types associated with benign genital and cervical papillomas which rarely progress to cancer are referred to as "low risk" (HPV-6 and -11) (1). Viral oncoproteins E6 and E7 encoded by the high-risk HPVs are expressed consistently in cervical cancers and disrupt regulations of the cell cycle (2). To understand carcinogenic functions of these oncoproteins, it is required to identify their cellular targets. E7 makes complexes with the retinoblastoma gene product (Rb) and the cdk inhibitors ($p21^{WAF1}$ and $p27^{KIP1}$) (3-6) and abrogates their functions resulting in an unscheduled entry into S phase without proliferative signals. E6 binds to tumor suppressor protein p53 and degrades it in cooperation with the E6-AP protein through a ubiquitin-mediated pathway (7). E6 can associate with E6BP, a putative calcium-binding protein (ERC-55) (8), paxillin (9), and hDlg/SAP97, a human homologue of the *Drosophila* discs large tumor suppressor protein (10, 11).

In this study, an attempt was made to detect unknown cellular protein(s) that HPV-16 E6 can bind. We screened a HeLa cell cDNA library by a yeast two-hybrid system using the N-terminal region of HPV-16 E6 as a bait and found that E6 made a complex with the C-terminal region of a human minichromosome maintenance 7 (hMCM7) protein (12) that plays a role in the licensing of DNA replication, a mechanism to ensure a single round of cellular DNA replication per cell cycle, in cooperation with the other MCM family proteins (13). We compared the binding of E6 with hMCM7 between the high-risk and low-risk HPVs.

MATERIALS AND METHODS

Yeast two-hybrid system. Expression plasmid pAS2-1 for a fusion protein with the GAL4 DNA-binding domain (GAL4-DBD), a HeLa cell cDNA library cloned into pGAD-GH that expresses cDNAs as fusion proteins with the GAL4 activation domain(GAL4-AD), yeast strains Y187 (*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met, gal80Δ, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*) and Y190 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh*^{r}*2, LYS2::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS3, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*) were purchased as MATCHMAKER Two-Hybrid System 2 from CLONTECH Laboratories, Inc. (Palo Alto, CA). The expression of *lacZ* gene in Y187 and those of *HIS3* and *lacZ* genes in Y190 are under the control of a GAL4 transcription factor. The transcription factor functionally reconstructed by binding of the two target proteins in yeast cells was assayed from measurement of β-galactosidase activities (*lacZ* expression) or from colony formation after 5 day period in the absence of histidine using agar medium containing 20 mM 3-amino-1, 2, 4-triazole (3-AT), a competitive inhibitor of residual *HIS3* protein.

For measuring the β-galactosidase activities, the yeast strain Y190 or Y187 was co-transfected with plasmids expressing two test proteins fused with GAL4-DBD and with the GAL4-AD. An overnight culture of transformed yeast single colony in leucine-tryptophan

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dropout medium was diluted to OD_{600} of 0.6 and cultured for additional 2-hr. Cells were harvested and lysed by freeze-thaw in a buffer consisting of 100 mM sodium phosphate (pH 7.1), 10 mM KCl, 1 mM $MgSO_4$, and 0.5 mM dithiothreitol. The β-galactosidase activities of the lysates were measured with Galacto-Star chemiluminescent assay kit (TROPIX, Inc., Bedford, MS) and normalized to the protein concentration of the lysates.

In vitro pull down assay. Full-length E6s fused with a maltose-binding protein (MBP-E6) were expressed in *E. coli* JM109 using pMAL-c2 (New England Biolabs, Inc.) and purified by amylose affinity chromatography. hMCM7 protein was translated and [^{35}S]-methionine-labeled in vitro in rabbit reticulocyte lysate (Promega) using RNA transcribed from pGEM-4Z (Promega) containing cDNA encoding full-length hMCM7. The reaction mixtures for in vitro translation of hMCM7 were diluted to 1:50 in a buffer [50 mM Tris(pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, pepstatin A and leupeptins (2 μg/ml)], incubated with MBP-E6 for 60 min at 4°C, and then mixed with amylose resin. The resin-bound proteins were separated by centrifugation and analyzed with 12.5% SDS-polyacrylamide gel electrophoresis. Labeled proteins in the gel were visualized by an image analyzer BAS5000 (Fuji Photo Film, Tokyo, Japan).

Immunoblotting. Proteins in yeast cells transformed with the expression plasmids for the two fusion proteins were extracted in a buffer containing 8 M urea and 5% SDS (14), separated by 12.5% SDS-polyacrylamide gel electrophoresis, blotted onto Hybond-P PVDF membrane (Amersham). The fusion proteins were visualized by ECL plus detection system (Amersham) using anti-GAL4-DBD and anti-GAL4-AD monoclonal antibodies (CLONTECH Laboratories, Inc.).

RESULTS

Cloning of cDNA encoding hMCM7. We screened a HeLa cDNA library by a yeast two-hybrid system using the region of amino acids (aa) 1 to 91 of HPV-16 E6 as a bait. After screening 2×10^6 clones by colony formation of Y190 in the absence of histidine, we isolated a cDNA encoding the C-terminal region (aa 563 to 719) of hMCM7 protein (12). The cDNA encoding the full-length hMCM7 protein was newly constructed by RT-PCR using two primers (nucleotides 111 to 129 for sense primer and 2289 to 2271 for anti-sense primer, both primers were flanked with *Eco*RI sites at 5′ ends) (12) and HeLa cell mRNA. The nucleotide sequence of the constructed cDNA was identical with the reported sequence except for C at nucleotide 547 (12).

In vitro binding of HPV-16 E6 to hMCM7. The association of E6 to full-length hMCM7 was shown by an in vitro pull down assay detecting the specific binding of ^{35}S-labeled, in vitro translated hMCM7 to MBP-E6 fusion proteins (FIG. 1). E6s of HPV-16, -18, and -6 bound to hMCM7 in vitro under the same conditions where Rb binds to hMCM7 (15). Approximately 1% of the mixed ^{35}S-labeled hMCM7 was precipitated specifically with E6s or Rb. Although the data indicate the binding of E6 to hMCM7 was comparable to the binding of Rb to hMCM7, the level of the binding was not high enough for quantitative analyses. In the following experiments, the two-hybrid system monitored by expression of *lacZ* or *HIS3* gene was used for detection of the binding.

Regions necessary for the complex formation. The interaction between the N-terminal region of HPV-16 E6 and the C-terminal region (aa 572 to 719) of hMCM7 protein (hMCM7-c) was verified by measuring β-galac-

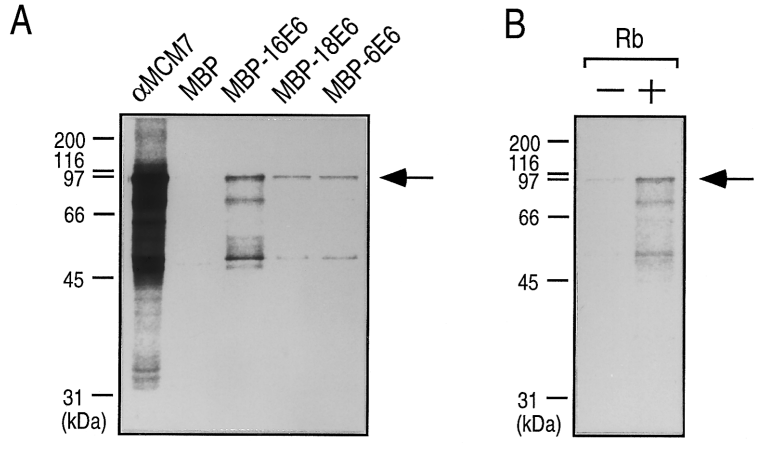

FIG. 1. (A) Association of HPV E6s with hMCM7 *in vitro.* Aliquots of the rabbit reticulocyte lysate programmed for production of full-length hMCM7 labeled with [^{35}S]methionine were incubated with MBP or MBP-E6s; MBP-16E6, MBP-18E6, and MBP-6E6 are full-length E6 proteins of HPV-16, -18, and -6 fused with MBP, respectively. For comparison, an aliquot of each lysate was incubated with anti-hMCM7 antiserum from a rabbit immunized with bacterially expressed protein in the C-terminal region (aa 519 to 719) of hMCM7 (αMCM7). The immunoprecipitate and amylose resin-bound E6 proteins were electrophoresed on a 12.5% SDS–polyacrylamide gel. Molecular mass markers are indicated to the left. (B) Association of Rb with hMCM7 *in vitro.* Rabbit reticulocyte lysates programmed for hMCM7 production with [^{35}S]methionine were incubated for 60 min at 4°C with (+) or without (−) human Rb protein ($p110^{RB}$) (QED Bioscience Inc., San Diego, CA). Rb protein was immunoprecipitated with anti-Rb monoclonal antibody 3H9 (Medical & Biological Laboratories, Nagoya, Japan) and resolved on a 12.5% SDS–polyacrylamide gel. Arrows indicate the position of full-length hMCM7.

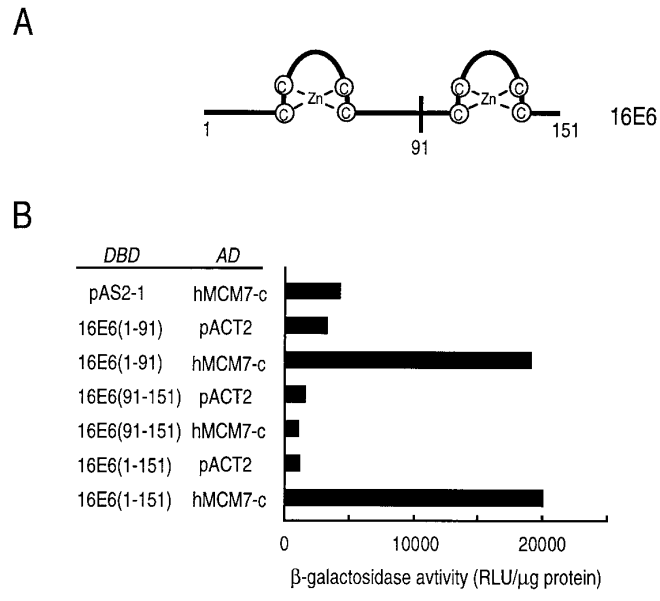

FIG. 2. N-terminal zinc-finger region of HPV-16 E6 bound to hMCM7. (A) A schematic representation of the HPV-16 E6 protein that contains two Zn-finger structures. The N-terminal (aa 1 to 91), the C-terminal (aa 91 to 151), and whole (aa 1 to 151) E6 were examined. (B) The β-galactosidase activities of the extracts from the yeast cells (strain Y187) containing two expression plasmids for E6s and hMCM7. pAS2-1 and pACT2 are the backbone plasmids for expression of GAL4-DBD and GAL4-AD, respectively. 16E6(1-91), 16E6(91-151), and 16E6(1-151) are the N-terminal, the C-terminal, and the whole HPV-16 E6 fused with GAL4-DBD, respectively. hMCM7-c is the C-terminal region (aa 572 to 719) of hMCM7 fused with GAL4-AD.

tosidase activities of extracts from Y187 containing both expression plasmids for E6 fused with GAL4-DBD and for hMCM7-c fused with GAL4-AD (FIG. 2). The data clearly show that the N-terminal region (aa 1 to 91) of E6 binds to hMCM7-c, whereas the C-terminal region (aa 91 to 151) does not.

The region of the hMCM7 necessary for binding with E6 was examined by a deletion analysis (FIG. 3). The β-galactosidase activities of Y187 expressing deleted hMCM7-c fused with GAL4-AD together with HPV-16 E6 fused with GAL4-DBD were measured. The data indicated that the essential region for the interaction was contained within the region from aa 602 to 719. The heptad K/R repeat sequence, which is conserved in all human MCM proteins (16), was necessary for the binding.

Bindings of E6 of HPV-16, -18, -58, -6 and -11 with hMCM7. The E6s of HPV-16, -18, and -58 (one of HPVs frequently found among Japanese patients with cervical cancer) bound with hMCM7-c more strongly than the E6s of HPV-6 and -11 did in the yeast cells. The interaction of E6s with hMCM7-c was examined in Y190 using medium containing 3-AT (FIG. 4A). The level of *HIS3* expression that is activated by the binding of GAL4-AD to GAL4-DBD can be estimated by the level of resistance to 3-AT. Although Y190 expressing any of the E6s together with hMCM7-c grew on the agar plates containing 25 mM of 3-AT, only the cells expressing E6s of HPV-16, -18 and -58 grew on the agar plates containing 50 mM of 3-AT.

The levels of β-galactosidase activities of extracts from Y190 were examined (FIG. 4B). The enzyme activities in the yeast cells containing E6s of HPV-16, -18, and -58 were higher than those in the cells containing the E6s of HPV-6 and -11. The binding of the HPV-18 E6 to the hMCM7-c was found to be the strongest. Similar results were obtained in the same assay using Y187 (data not shown).

The presence and stability of E6 fused with GAL4-DBD and hMCM7-c fused with GAL4-AD in Y190 were examined by immunoblot using anti-GAL4-DBD and anti-GAL4-AD monoclonal antibodies. Whereas hMCM7-c fused with GAL4-AD was found at a comparable level among the yeast clones (data not shown),

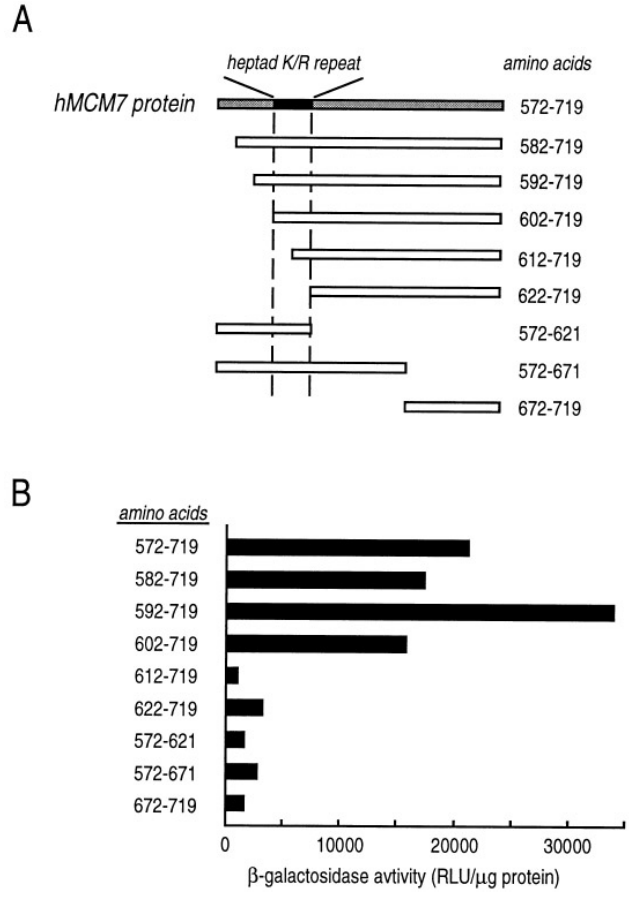

FIG. 3. C-terminal region (aa 602 to 719) of hMCM7 bound to E6. (A) A schematic representation of deleted hMCM7 proteins. (B) The β-galactosidase activities of the extracts from the yeast cells (strain Y187) expressing HPV-16 E6 and deleted hMCM7. The regions of hMCM7 fused with GAL4-AD are indicated by amino acids numbers.

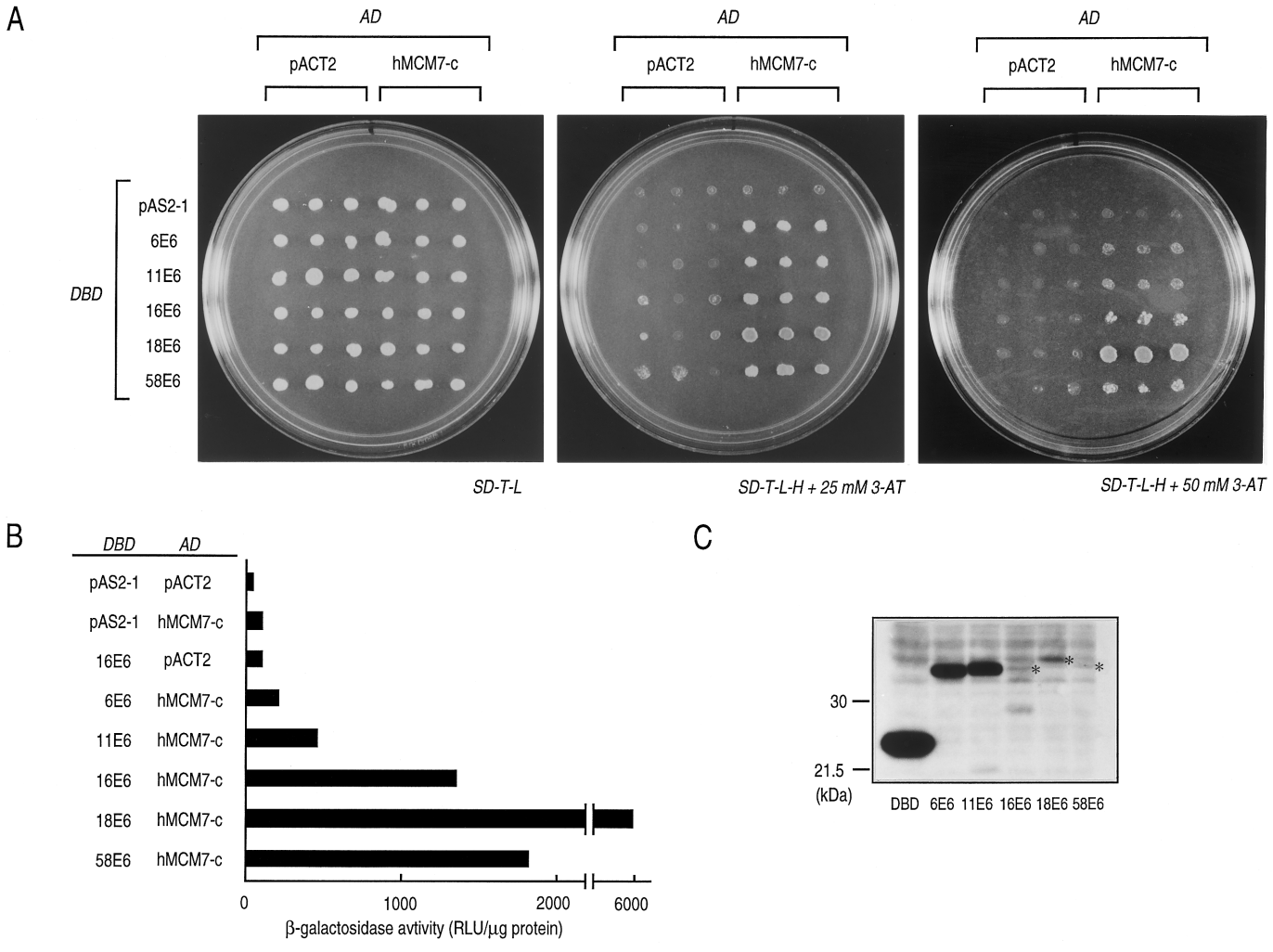

FIG. 4. Interaction of E6 of different HPVs with the C-terminal region of hMCM7. pAS2-1 is the backbone plasmid for expression of GAL4-DBD. 16E6, 6E6, 11E6, 18E6, and 58E6 are E6 proteins of HPV-16, -6, -11, -18, and -58 fused with GAL4-DBD, respectively. pACT2 is the backbone plasmid for expression of GAL4-AD. hMCM7-c is the C-terminal region (aa 572 to 719) of hMCM7 fused with GAL4-AD. (A) Growth of the yeast cells (strain Y190) co-transfected with expression plasmids for GAL4-DBD and for GAL4-AD on agar plates. (Left) Histidine (+). (Center) Histidine (−), 25 mM of 3-amino-1,2,4-triazole (3-AT) that is a competitive inhibitor of the yeast *HIS3* protein. (Right) Histidine (−), 50 mM of 3-AT. (B) The β-galactosidase activities of the extracts from the yeast cells (Y190) expressing E6s from various HPVs and hMCM7-c. (C) Immunoblotting detection of E6 proteins fused with GAL4-DBD using mouse monoclonal antibody against GAL4-DBD in the yeast cells (Y190) expressing hMCM7-c fused with GAL4-AD. The positions of 16E6, 18E6, and 58E6 are indicated with asterisks (*).

the levels of E6s fused with GAL4-DBD varied among HPV types. The steady-state levels of the E6s of HPV-6 and -11 were much higher than those of HPV-16, -18, and -58 (FIG. 4C). Although their significance is unclear now, the data indicate that the difference in the binding between the two HPV groups would be possibly greater than it appears as assayed with β-galactosidase activities (FIG. 4B), if the steady-state levels of E6s in yeast cells could be considered for comparison.

DISCUSSION

Recent studies have revealed the mechanisms how E6 and E7 overcome cell cycle control and apoptosis using p53 and Rb. However, it is still unclear whether they are involved in malignant phenotype of deregulated proliferative cells. We made an attempt to detect unknown cellular protein(s) that HPV-16 E6 can bind and found that E6 made a complex with the C-terminal region of a human minichromosome maintenance 7 (hMCM7) protein.

The MCM complex composed of human MCM2, 3, 4, 5, 6 and 7 associates with chromatin before the onset of replication (late mitosis and G1 phase) and are displaced during S phase (17-19). Block of re-association of the complex to chromatin is considered to ensure a single round of cellular DNA replication per cell cycle (20). Multiple cellular regulatory proteins including

cdc6 and cdks are supposed to regulate binding capacity of the MCM complex to chromatin (21).

The data obtained in this study suggest that the binding of E6 to hMCM7 may be related to the oncogenic potential of the HPVs associated with cervical cancer, but it remains to be studied how the binding affects the target cells. The N-terminal region of Rb binds to the C-terminal region (137 aa) of hMCM7 (15). The binding of human Rb to *Xenopus* MCM7 inhibits DNA replication in an MCM7-dependent manner in vitro, suggesting that the C-terminal region of MCM7 plays a regulatory role for licensing function of the MCM complex. Since E6-binding region (C-terminal 118 aa) is overlapping with the Rb-binding region, it is possible that the regulatory function of the MCM complex may be interfered by the bindig of E6. The disturbance of the accurate licensing mechanisms by the binding of E6 may cause chromosomal abnormalities observed in the cells expressing E6 of high-risk HPVs (22, 23) and integration of the HPV DNA into host cell DNA occurring in cervical cancer cells (24).

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REFERENCES

1. Lorincz, A. T., Reid, R., Jenson, A. B., Greenberg, M. D., Lancaster, W. and Kurman, R. J. (1992) *Obstet. Gynecol.* **79,** 328–337.
2. Kubbutat, M.H.G. and Vousden, K.H. (1996) *Semin. Virol.* **7,** 295–304.
3. Münger, K., Werness, B.A., Dyson, N., Phelps, W.C., Harlow, E., and Howley, P.M. (1989) *EMBO J.* **8,** 4099–4105.
4. Zerfass-Thome, K., Zwerschke, W., Mannhardt, B., Tindle, R., Botz, J. W., and Jansen-Durr, P. (1996) *Oncogene* **13,** 2323–2330.
5. Funk, J. O., Waga, S., Harry, J. B., Espling, E., Stillman, B., and Galloway, D. A. (1997) *Genes Dev.* **11,** 2090–2100.
6. Jones, D. L., Alani, R. M., and Münger, K. (1997) *Genes Dev.* **11,** 2101–2111.
7. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. (1990) *Cell* **63,** 1129–1136.
8. Chen, J. J., Reid, C. E., Band, V., and Androphy, E. J. (1995) *Science* **269,** 529–531.
9. Tong, X., and Howley, P. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4412–4417.
10. Lee, S. S., Weiss, R. S., and Javier, R. T. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6670–6675.
11. Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T., and Ishibashi, M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11612–11616.
12. Kiyono, T., Fujita, M., Hayashi, Y., and Ishibashi, M. (1996) *Biochim. Biophys. Acta* **1307,** 31–34.
13. Chong, J. P., Thommes, P., and Blow, J. J. (1996) *Trends Biochem. Sci.* **21,** 102–106.
14. Printen, J. A., and Sprague, G. F., Jr. (1994) *Genetics* **138,** 609–619.
15. Sterner, J. M., Dew-Knight, S., Musahl, C., Kornbluth, S., and Horowitz, J. M. (1998) *Mol. Cell. Biol.* **18,** 2748–2757.
16. Kubota, Y., Mimura, S., Nishimoto, S., Masuda, T., Nojima, H., and Takisawa, H. (1997) *EMBO J.* **16,** 3320–3331.
17. Todorov, I. T., Attaran, A., and Kearsey, S. E. (1995) *J. Cell Biol.* **129,** 1433–1445.
18. Krude, T., Musahl, C., Laskey, R., and Knippers, R. (1996) *J. Cell Sci.* **109,** 309–318.
19. Fujita, M., Kiyono, T., Hayashi, Y., and Ishibashi, M. (1997) *J. Biol. Chem.* **272,** 10928–10935.
20. Kearsey, S. E., Maiorano, D., Holmes, E. C., and Todorov, I. T. (1996) *Bioessays* **18,** 183–190.
21. Rowles, A., and Blow, J. J. (1997) *Curr. Opin. Genet. Dev.* **7,** 152–157.
22. Xiong, Y., Kuppuswamy, D., Li, Y., Livanos, E. M., Hixon, M., White, A., Beach, D., and Tlsty, T. D. (1996) *J. Virol.* **70,** 999–1008.
23. Kaufmann, W. K., Schwartz, J. L., Hurt, J. C., Byrd, L. L., Galloway, D. A., Levedakou, E., and Paules, R. S. (1997) *Cell Growth Differ.* **8,** 1105–1114.
24. zur Hausen, H. (1996) *Biochim. Biophys. Acta* **1288,** F55–78.