Inactivation of the tumor suppressor pRB by the human papillomavirus (HPV) oncoprotein E7 is a mechanism by which HPV promotes cell growth. The bovine papillomavirus type 1 (BPV-1) E7 does not bind pRB efficiently yet is required for full transformation of murine cells by BPV-1. In the present study, we investigated the mechanism of BPV-1 E7-induced cell proliferation. Our studies indicate that expression of BPV-1 E7 induces DNA synthesis and stimulates cells to enter S phase in quiescent cells. The induction of cell proliferation by BPV-1 E7 can occur in the retinoblastoma gene (Rb)-null cells, suggesting an Rb-independent mechanism. Consistent with this observation, BPV-1 E7 does not efficiently activate the transcription of the E2F family of transcription factors (E2F)-responsive promoters. Notably, c-Myc is able to induce cells to enter S phase in quiescent cells through an Rb/E2F-independent pathway. Significantly, c-Myc levels are increased in BPV-1 E7-expressing cells. Moreover, expression of a dominant negative c-Myc mutant inhibited BPV-1 E7-induced DNA synthesis. Consistent with the notion that c-Myc could down-regulate p27 and activate Cdk2, p27 level is decreased while both cyclin A and cyclin E-associated kinase activities are up-regulated in BPV-1 E7-expressing cells. These studies indicate an important role for c-Myc in BPV-1 E7-induced cell proliferation.

Papillomaviruses are small DNA viruses that replicate in the stratified layers of skin and mucosa and usually give rise to benign lesions such as warts or papillomas. Some animal papillomaviruses, including BPV-1, induce fibropapillomas. Because of its ability to transform cells and replicate its genome in established murine cell lines, BPV-1 has served as the prototype for studies of molecular biology of the papillomaviruses (for review, see Ref. 1). Specific types (“high risk”) of HPV's infect the anogenital tract and are strongly associated with the development of cervical carcinoma (for review, see Ref. 2).

The transforming properties of high risk HPV's primarily reside in E6 and E7 genes. HPV E7 cooperates with E6 to efficiently immortalize primary human epithelial cells (reviewed in Ref. 3). High risk HPV E7 induces DNA synthesis in quiescent or differentiated cells and transforms immortalized rodent cells (Refs. 4 and 5 and references therein). The E7 proteins of both the low and high risk HPV's were able to activate the Ad E2 promoter (6–8). HPV-16 E7 has the ability to overcome p21−, p27−, and p53-mediated cell cycle arrest and potentiates tumorigenesis in transgenic mice (reviewed in Ref. 3). Recently, a role in maintenance of epimastig during the viral life cycle and modulation of cellular response to apoptosis by HPV-16 E7 has been reported (reviewed in Ref. 3).

The ability of E7 protein to associate with and destabilize the cellular tumor suppressor pRB has been suggested as a mechanism by which the viral protein promotes cell proliferation (Ref. 9 and references therein). Inactivation of pRB leads to activation of cellular genes driven by the E2F transcription factor that are important for S phase entry in mammalian cells (reviewed in Ref. 10). However, pRB-independent biological activities of E7 have been observed and multiple additional cellular interactors of the viral proteins have been identified (for review, see Ref. 3).

The major transforming proteins encoded by BPV-1 are E5 and E6. Each of these proteins is sufficient to induce transformation of murine C127 cells (11–15). Although no independent oncogenic activity has been detected for BPV-1 E7, it was shown to be required for efficient transformation of C127 by BPV-1 (16). It was suggested that the transforming capability of BPV-1 E7 was repressed by other viral genes in the context of BPV-1 genome (17). We have recently shown that expression of BPV-1 E7 sensitizes cells to tumor necrosis factor-α-induced apoptosis in the absence of Rb (18). In addition, conflicting data have been published on the role of BPV-1 E7 in BPV-1 genome copy number regulation (Ref. 19 and references therein).

With the exception of the Cys-X-X-Cys motifs, the E7 proteins of the BPV-1 and the HPVs are quite different in their amino acid composition (<20% identity). BPV-1 E7 lacks the sequence motif (LXCXE) present in the HPV E7 proteins, which is critical for efficient binding to the Rb family proteins (20, 21). However, the sequences in the C-terminal half of E7 proteins are well conserved between BPV-1 and HPVs. A low affinity pRB-binding site has been identified from the C terminus of HPV-16 E7 (22). We have demonstrated a low affinity association of BPV-1 E7 protein with pRB but not p107 (18).

In this study, we investigated the function and mechanism of BPV-1 E7 in cell proliferation. Our results indicate that BPV-1 E7 can stimulate cells to enter S phase in the absence of Rb.
Furthermore, c-Myc plays an important role in the ability of BPV-1 E7 to activate cyclin A- and cyclin E-associated kinases.

MATERIALS AND METHODS

Plasmids and Cell Culture—The Ad E2 reporter pAdE2lac contains the Ad E2 promoter followed by the sequences encoding lacZ (25). The pHBl-CMV reporter contains the CMV immediate-early enhancer/ promoter region followed by a synthetic Renilla gene sequence (Promega). The dihydrofoleate reductase (DFHR)-luciferease contains murine DFHR promoter followed by the luciferease cDNA (24). pLTRE encodes HPV-16 E7 downstream of the Harvey murine sarcoma virus LTR (25). Similarly, pLTRBE7 encodes BPV-1 E7 downstream of the Harvey murine sarcoma virus LTR. pCMVMadMyc encodes a dominant negative mutant of c-Myc (26). pDsRed1-N1 (Clontech, Inc., Palo Alto, CA) encodes a red fluorescent protein (RFP). pEGFP contains a farnesylated GTP (27).

PB7N cells were established by infection of NIH3T3 cells with pBabe Puro-based retroviruses encoding BPV-1 E7 (18). Control PU- RON cells contain the pBabe Puro vector. Similarly, PB7RB and PURON cells were established by infection of Rb÷ mouse embryo fibroblast from embryo taken at gestational day 12 (28) with pBabe Puro-based retroviruses encoding BPV-1 E7 and the retrovirus vector, respectively.

PCR—Reverse transcriptase reaction was performed with 1 μg of total cellular RNA isolated from various cell lines used as a template to synthesize cDNA using SuperScript II reverse transcriptase and an total cellular RNA isolated from various cell lines used as a template to synthesize cDNA using SuperScript II reverse transcriptase and an oligo(dT) primer (Invitrogen). The PCR reaction was carried out with TaqDNA polymerase (Promega). BPV-1 E7-specific primers (sense, nucleotides 3–20 (5′-GGTTCAAGGTCTTCAAAC-3′); antisense, nucleotides 171–152 (5′-TAGTGGGACTCGCTTCCTA-3′)) were used to amplify a 171-nucleotide fragment from the E7 cDNA. HPV-16 E7-specific primers (sense, nucleotides 3–20 (5′-CGCATGAGGTCCGAGAATCC-3′); antisense, nucleotides 122–102 (5′-GAGCACTCATTTCTACGCTC-3′)) were used to amplify a 124-nucleotide fragment from the HPV-16 E7 cDNA. c-Myc-specific primers (sense, nucleotides 906–923; antisense, nucleotides 1295–1271 (5′-CGCTGTGGCTTCTCAATAGCTC-3′)) were used to amplify a 390-nucleotide fragment from the c-Myc cDNA. For β-actin, the sense primer 5′-TGGCATGCGCCAGAGTTGAGAA-3′ and the antisense primer 5′-CTCGTCACTCCTCGGTATG-3′ were used to amplify a 172-nucleotide fragment.

Flow Cytometry—Cells were seeded in 60-mm dishes with Dulbecco’s minimum essential medium (DMEM) plus 10% fetal calf serum at 2 × 105 cells/dish. The following day, the DMEM was changed to medium with 0.5% fetal calf serum (serum-starved) and incubated for 48 h. Cells were harvested by trypsinization and fixed in 70% ethanol overnight. Cells were washed four times with lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors including aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). 100,000 cells of total protein extract were immunoprecipitated with appropriate antibody and protein A-agarose (Amersham Biosciences). The beads were washed twice with lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, and protease inhibitor cocktail (Sigma)). Assays were performed in the presence of 10 μCi of [32P]ATP (30 Ci/mmol, New England Nuclear) and 0.1 mM ATP for 30 min at 30 °C. 2 μg of histone H1 (Sigma) was used as the substrate in the kinase assays. Following the kinase reaction, samples were boiled in Lamelli sample buffer, separated by 12% SDS-polyacrylamide gel electrophoresis. Phosphorylated proteins were visualized by autoradiography.

RESULTS

Expression of BPV-1 E7 Induces Cells to Enter S Phase—To study the biological activities of BPV-1 E7, mouse NIH 3T3 cells expressing BPV-1 E7 and control vectors named PB7N and PURON, respectively, were established. For this study, NIH3T3 cells were infected with amphotropic retrovirus-expressing BPV-1 E7. After puromycin selection, populations of infected cells were pooled. Similarly, the Rb−/− mouse embryonic fibroblast-expressing BPV-1 E7 (PB7RB) and control cells containing the retrovirus vector (PURON−/−) were also established. BPV-1 E7 gene expression was confirmed by PCR amplification of the cDNA after reverse transcription of cellular mRNA (Fig. 1). Morophilologically, there are no significance differences between cells expressing BPV-1 E7 and the vector control (data not shown). To avoid the possibility of chromosomal instability due to the expression of BPV-1 E7, all of the experiments were performed using cells within three passages. To investigate the effect of BPV-1 E7 on cell cycle progression, PB7N and control PURON cells were serum-starved and analyzed for DNA content by flow cytometry. The result of a representative experiment is shown in Fig. 1B. Clearly, a significant proportion of the E7-expressing cells entered S phase, whereas control cells largely remained arrested in the G1 phase under serum starvation. No significant difference in percentage of S phase between PB7N and PURON cells was observed when they were cultured in 10% fetal calf serum. Increased S phase of unsynchronized cells by BPV-1 E7 could be the result of its induction of cells to enter S phase or prevention of cell cycle arrest from occurring. To demonstrate that BPV-1 E7
indeed has the ability to promote quiescent cells to enter the cell cycle, we treated PBE7N cells with the potassium/sodium channel blocker quinidine to arrest cells in G1 (30) and asked whether cells expressing BPV-1 E7 can enter the cell cycle when they were released into low serum medium lacking quinidine. Flow cytometry analysis showed that although control PURON cells that were released from a quinidine block into low serum media for 24 h failed to enter S phase, PBE7N cells did enter S phase (Fig. 1C). These results demonstrate that BPV-1 E7 can induce S phase entry, although we could not rule out the possibility that it may also have the ability to prevent cell cycle arrest from occurring. This function of BPV-1 E7 probably mimics its role to induce DNA synthesis in terminally differentiated cells.

The ability of BPV-1 E7 to promote cell cycle progression was further analyzed by measuring BrdUrd incorporation. BrdUrd incorporates into newly synthesized DNA strands of actively proliferating cells. After serum starvation of PBE7N and PURON cells, BrdUrd was added to the culture and was detected immunohistochemically. As shown in Fig. 2A, cells expressing BPV-1 E7 had significantly higher BrdUrd incorporation (2-fold higher) than the control cells. No significant difference in BrdUrd incorporation between PBE7N and PURON cells was observed when they were cultured in 10% fetal calf serum (data not shown).

Induction of Cell Proliferation by BPV-1 E7 Can Occur in the Absence of Rb—Although our previous study showed that...
BPV-1 E7 did not bind pRB efficiently \textit{in vitro} or promoted its degradation \textit{in vivo} (18), it remains possible that inactivation of Rb is required for BPV-1 E7 to induce cell proliferation. To assess the role of Rb in this process, PBE7R/B–/– and control cells were examined for BrdUrd incorporation after serum starvation. As shown in Fig. 2B, significantly elevated BrdUrd incorporation was observed in cells expressing BPV-1 E7 as compared with the control cells. These data indicate that BPV-1 E7-induced cell proliferation can occur in the absence of Rb, although it does not rule out the possibility that some of the activities seen in PBE7N cells involve Rb inactivation.

The E7 proteins of both the low and high risk HPVs were found to be able to activate the E2F-responsive Ad E2 promoter (6–8, 31). In some cells, however, the ability of HPV E7 to activate the Ad E2 promoter correlated with its binding efficiency to pRB (31). This promoter contains E2F-binding sites whose activation relies on functional inactivation of Rb and Rb family members. Therefore, we assessed the ability of BPV-1 E7 to activate the transcription of Ad E2 promoter. Plasmid expressing BPV-1 E7 was co-transfected with an Ad E2 luciferase reporter plasmid (23) into NIH3T3 cells, and luciferase activities were determined. Although our positive control HPV-16 E7 activated the Ad E2 promoter for nearly 4-fold, BPV-1 E7 did not efficiently increase the transcription of the reporter (<0.2-fold increase, see Fig. 3). The control assay with the CMV-\textit{Renilla} luciferase reporter demonstrated that the failure of stimulation by BPV-1 E7 is not because of a lack of efficient transfection. RT-PCR analysis indicated similar levels of HPV-16 E7 and BPV-1 E7 mRNAs (data not shown). Therefore, defective Ad E2 promoter activation by BPV-1 E7 cannot be attributed to the level of its expression.

To ascertain that the lack of efficient activation of E2F-responsive promoter by BPV-1 E7 is not restricted to Ad E2, we examined the ability of BPV-1 E7 to activate another E2F-responsive promoter, the \textit{DHFR} promoter. It was shown that E2F binding is required to activate the \textit{DHFR} promoter (32, 33). Accordingly, plasmids encoding E7 were co-transfected with a DHFR luciferase reporter plasmid into NIH3T3 and luciferase activities were determined. While HPV-16 E7 activated \textit{DHFR} reporter for severalfold, BPV-1 E7 did not alter the reporter activity (data not shown). Taken together, results from our studies indicate that Rb is not a major target for BPV-1 E7. Our studies with the E2F-responsive promoters also suggest that the Rb family members p107 and p130 may not play an important role in BPV-1 E7-induced cell proliferation as they also repress E2F-regulated promoters (34).

\textbf{Activation of c-Myc Contributes to BPV-1 E7-induced Cell Proliferation}—Because BPV-1 E7 could induce cell cycle progression in the absence of Rb, we began to investigate Rb-independent pathways by which BPV-1 E7 performs its functions. It was shown that ectopic expression of c-Myc was able to induce cells to enter S phase in quiescent cells (reviewed in Ref. 35). Moreover, c-Myc activity was shown to be involved in a G1/S-promoting mechanism by regulating cyclin E-Cdk2 function that is independent of E2F and parallel to the Rb/E2F pathway (36). The pRB-independent induction of cell proliferation by BPV-1 E7 raises the possibility that E7 activates c-Myc. Therefore, we investigated whether BPV-1 E7 could induce c-Myc expression. Accordingly, extracts were prepared from PBE7N and control cells and levels of c-Myc were examined by Western blot. As shown in Fig. 4A, cells expressing BPV-1 E7 exhibited a significantly increased level of c-Myc (~4-fold) in NIH3T3 cells after serum starvation. Furthermore, BPV-1 E7 expression also increased c-Myc level to a less extent under normal culture conditions (i.e. DMEM with 10% fetal calf serum) where no difference in cell proliferation was observed between BPV-1 E7 and control cells. These data suggest that the elevated levels of c-Myc in E7-expressing cells are not simply a result of increased cell proliferation. Up-regulation of c-Myc in RB–/– cells expressing BPV-1 E7 was also observed (data not shown).

To determine whether BPV-1 E7 induces c-Myc by increasing its mRNA, we performed RT-PCR assays. As shown in Fig. 4B, c-Myc mRNA is increased for >3-fold in PBE7N cells as compared with PURON cells. Similarly, c-Myc mRNA is also increased in PBE7R/B–/– cells. Although this result does not rule out the possibility that BPV-1 E7 may also regulate c-Myc by post-transcriptional mechanism, it suggests that BPV-1 E7 could activate the transcription of the \textit{c-myc} gene.

To further establish the role of c-Myc in BPV-1 E7-induced cell proliferation, we expressed a dominant negative mutant of c-Myc (MadMyc) in PBE7N cells and cellular DNA replication was assessed by BrdUrd incorporation. MadMyc is a chimeric protein that contains the DNA binding/dimerization domains of c-Myc and transcriptional repression domain of Mad (26). It is believed to cause repression of c-Myc-responsive genes by binding to the c-Myc-binding elements. Transient expression of MadMyc induced G1 arrest independent of functional Rb (26, 37). Accordingly, an RFP-expressing vector was co-transfected with MadMyc or vector control to PBE7N cells. After transfection, cells were serum-starved and BrdUrd was added to the culture for the final 8 h. Cells were then immunostained for BrdUrd and scored for BrdUrd incorporation. Fig. 4C shows that MadMyc expression inhibited BPV-1 E7-induced DNA synthesis. In MadMyc-expressing PBE7N cells, the number of BrdUrd-positive cells was reduced by >50%. These results indicate an important role of c-Myc in the induction of cell proliferation by BPV-1 E7.

\textbf{Down-regulation of p27 and Up-regulation of Cyclin A and Cyclin E-associated Kinase Activities in BPV-1 E7-expressing Cells}—The mechanism of c-Myc-induced cell proliferation is complex including the up-regulation of Cdc25A, cyclin A, cyclin D1, cyclin E, and down-regulation of p27 (reviewed in Ref. 35). These pathways converge on the control of Cdk2 activity, which is rate-limiting and essential for DNA replication. To investigate which of these protein levels altered in BPV-1 E7-expressing cells, we performed Western blot analysis. Accordingly, extracts were prepared from PBE7N and control PURON cells.
As shown in Fig. 5A, cells expressing BPV-1 E7 exhibited a decreased level of p27 (1.4-fold) under serum starvation. This result is consistent with the observation that p27 is a critical G1 phase cell cycle target of c-Myc (38). In contrast, no significant difference was observed for Cdc25A, Cdk2, cyclin A, cyclin D1, and cyclin E protein levels between PBE7N and control PURON cells.

We next examined Cdk2 kinase activities in BPV-1 E7-expressing cells. For this goal, cyclin A, cyclin D, and cyclin E were immunoprecipitated from PBE7N, PBE7RB/−/−, and control cell extracts with specific antibodies and the respective kinase activities with histone H1 were determined. As shown in Fig. 5B, PBE7N cells showed a significant increase in cyclin E-associated kinase activity (2.5-fold). Cyclin A-associated kinase activity was also increased in PBE7N cells (1-fold). Similar increased cyclin A and cyclin E-associated kinase activities were also observed in Rb−/− cells expressing BPV-1 E7 (data not shown). In contrast, the cyclin D-associated kinase activity in PBE7N cells was decreased (2-fold). The reason for the reduced cyclin D-associated kinase activity in PBE7N cells is not known. Nevertheless, cyclin E can induce S phase entry without activation of E2F-dependent transcription or pRB phosphorylation (39–42) and ectopic expression of cyclin A also allows S phase entry of rodent fibroblasts (43).

DISCUSSION

Inactivation of the tumor suppressor pRB by the HPV E7 is a mechanism by which HPV promotes cell growth. pRB-independent biological activities of E7 have been observed, but the precise mechanism for these E7 functions are not well understood. The BPV-1 E7 does not bind pRB efficiently yet is required for full transformation of murine cells by BPV-1. The BPV-1 E7 thus provides a good model to explore Rb-independent activities of E7. In this study, we provided evidence that BPV-1 E7-induced cell proliferation can occur in the absence of Rb. Our studies demonstrate that activation of c-Myc contributes to BPV-1 E7-induced cell proliferation. Furthermore, down-regulation of p27 by c-Myc resulted in the activation of cyclin A and cyclin E-associated kinases. These studies indicated an important role of c-Myc in BPV-1 E7-induced cell proliferation.

c-Myc is involved in many biological activities including in-
BPV-1 E7 Activates c-Myc to Stimulate S Phase Entry

Consistent with the finding of previous studies, we observed that HPV-16 E7 induces a decrease in p27 levels (38, 49), which has been attributed to E7-induced down-regulation of the Cdk inhibitor p27 (38, 49). Further studies are needed to determine whether the activity of the Cdk inhibitor p27 (38, 49) raises the possibility that BPV-1 E7 may down-regulate p27 to activate c-Myc. However, our data that c-Myc protein level is unchanged significantly in our study.

Although it is well established that c-Myc down-regulates p27, a recent study suggests that p27 could also inhibit c-Myc expression and cyclin E-Cdk2 activity (52). This observation raises the possibility that BPV-1 E7 may down-regulate p27 to activate c-Myc. However, our data that c-Myc protein level is increased in both serum-starved and non-starved cells while down-regulation of p27 only occurs in serum-starved BPV-1 E7-expressing cells suggest that p27 is downstream of c-Myc in the activation of Cdk2 activities in these cells. Notably, although cyclin D level was slightly increased, the cyclin D-associated kinase activity in PBV-1 E7-expressing cells was decreased. A likely explanation for this observation is that the sequestration of p27 into cyclin D-Cdk4 complexes away from cyclin E-Cdk2 by c-Myc (53). However, this process is complex as one study found that p27 acted as an activator of cyclin D-dependent kinase activity (54). Further studies are required to understand the mechanism and significance of cyclin D-dependent kinase down-regulation in BPV-1 E7-expressing cells.

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