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The human papillomavirus type 16 E7 gene product interacts with and *trans*-activates the AP1 family of transcription factors

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The E7 gene product of human papillomavirus type 16 (HPV16) binds to the retinoblastoma gene product (pRb) and dissociates pRb–E2F complexes. However, the observation that the ability of E7 to bind pRb is not required for the HPV16-induced immortalization of primary keratinocytes prompted a search for other cellular factors bound by E7. Using a glutathione-*S*-transferase (GST) fusion protein system, we show that E7 complexes with AP1 transcription factors including c-Jun, JunB, JunD and c-Fos. The ability of E7 to complex with c-Jun *in vivo* is demonstrated by co-immunoprecipitation and the yeast two-hybrid system. An analysis of E7 point mutants in the GST system indicates that the E7 zinc-finger motif, but not the pRb binding domain, is involved in these interactions. Using c-Jun deletion mutants, E7 binding maps between amino acids 224 and 286 of c-Jun. E7 *trans*-activates c-Jun-induced transcription from a Jun responsive promoter, and this activity correlates with the ability of E7 mutants to bind Jun proteins. Finally, a transcriptionally inactive c-Jun deletion, which can bind E7, interferes with the E7-induced transformation of rat embryo fibroblasts in cooperation with an activated *ras*, indicating that the Jun–E7 interaction is physiologically relevant and that Jun factors may be targeted in the E7 transformation pathway.

Keywords: AP1/E7/human papillomavirus/Jun

Introduction

The human papillomaviruses (HPVs) which infect the anogenital tract have been divided into two groups based on their association with malignant disease. HPV6 and HPV11 are representative of the low-risk group which are most commonly associated with condyloma acuminata (or genital warts) and low-grade cervical intraepithelial neoplasia (CIN; Gissman *et al.*, 1983; McCance *et al.*, 1986). HPV16 and HPV18 are members of the high-risk group which are often present in high-grade CINs and malignant cervical disease (Durst *et al.*, 1983; Boshart *et al.*, 1984; McCance *et al.*, 1985). The distinction between these two groups has been extended in tissue culture models, where HPV16 and HPV18 have been shown to transform rodent fibroblasts and immortalize primary human keratinocytes, while types 6 and 11 show weak rodent cell-transforming potential and no human keratinocyte-immortalizing activity (Pirisi *et al.*, 1987; McCance *et al.*, 1988; Chesters and McCance, 1989; Woodworth *et al.*, 1989). Of the HPV16- and HPV18-encoded genes, E6 and E7 have been demonstrated to be necessary and sufficient for both of these activities (Bedell *et al.*, 1987; Vousden *et al.*, 1988; Münger *et al.*, 1989; Hudson *et al.*, 1990; Halbert *et al.*, 1991).

The E7 reading frame of HPV16 encodes a 98 amino acid protein able to transform NIH 3T3 cells (Banks *et al.*, 1990) and cooperate with an activated *ras* or c-*fos* to transform rat embryo fibroblasts (REFs; Chesters and McCance, 1989; Chesters *et al.*, 1990). E7 is phosphorylated on two serine residues by casein kinase II, or CKII (Barbosa *et al.*, 1990), and contains two cysteine–X–X–cysteine motifs believed to form a zinc finger (Barbosa *et al.*, 1989). At the molecular level, the transforming and immortalizing activity of E7 has been mainly attributed to the ability of E7 to bind to the retinoblastoma protein (pRb) through regions homologous to the adenovirus E1A and simian virus 40 large tumor antigen pRb binding domains (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989). The regions of homology include conserved regions 1 and 2 (CR1 and CR2), present in both the 12S and 13S forms of E1A (Phelps *et al.*, 1988). The low-risk HPV types 6 and 11 E7 proteins exhibit a lower binding affinity for pRb than the HPV16 and 18 E7s (Gage *et al.*, 1990), and the abrogation of pRb binding by site-directed mutagenesis interferes with the ability of HPV16 E7 to transform NIH 3T3s and REFs (Edmonds and Vousden, 1989; Chesters *et al.*, 1990). E7 has also been shown to associate with the pRb-related p107 protein, an H1 kinase activity (Davies *et al.*, 1993; Tommasino *et al.*, 1993), and cyclin A (Tommasino *et al.*, 1993), but the significance of these interactions is not yet understood.

Transcriptional activation by the adenovirus E1A gene products has been studied extensively (for a review see Nevins, 1993) and may provide an insight into the mechanisms of E7 transcriptional activation. Like E7, the adenovirus E1A protein *trans*-activates the adenovirus E2 promoter, and the *trans*-activation is dependent on the E2F and activating transcription factor (ATF) recognition sequences within the promoter (Phelps *et al.*, 1988, 1991). The ability of E1A to *trans*-activate E2F-mediated transcription maps to the CR1 and CR2 regions of E1A, both of which are involved in pRb binding (Bagchi *et al.*, 1990). This observation led to the discovery that E2F is normally inactivated in the G_1 phase of the cell cycle by complex formation with pRb or the closely related p107 protein (Shirodkar *et al.*, 1992). At the G_1/S transition, pRb undergoes cell cycle-regulated hyperphosphorylation, which causes the dissociation of the E2F–pRb complex, giving rise to transcriptionally active free E2F. Recent

observations have also begun to elucidate the mechanism of the E1A-mediated *trans*-activation of ATF recognition sites. E1A was described to complex with ATF-2 as well as members of the AP1 family of transcription factors, including c-Jun and c-Fos, *in vitro* (Maguire *et al.*, 1991; Liu and Green, 1994). The formation of complexes containing E1A and c-Jun or ATF-2 involves the CR3 region of the 13S E1A gene product and the DNA binding region of c-Jun or ATF-2 (Liu and Green, 1994). E1A induces the formation of c-Jun–ATF-2 heterodimers, which represent a transcriptionally active complex specifically targeted by E1A (van Dam *et al.*, 1993). This activity does not appear to be totally independent of the CR1 and CR2 regions, as the 12S E1A gene product can activate ATF sites within the PCNA promoter (Morris and Mathews, 1991) and can either repress or *trans*-activate transcription from AP1 recognition sites in a CR1-dependent manner (Offringa *et al.*, 1990; Hagmeyer *et al.*, 1993). It is interesting to note that the E1A CR3 region contains a zinc-finger motif (Culp *et al.*, 1988), as does the C-terminus of E7 (Barbosa *et al.*, 1989).

We have demonstrated previously that, in the context of the entire HPV16 genome, pRb binding by E7 is not required for the immortalization of primary human keratinocytes (Jewers *et al.*, 1992). However, the disruption of one Cys–X–X–Cys motif in E7 abolished the HPV16-mediated keratinocyte immortalization in repeated experiments. Although pRb binding may still be required for the development of invasive disease, these results indicate that E7 encodes an immortalizing function separate from pRb binding. To investigate this function further, we have examined the ability of HPV16 E7 to bind to other cellular factors using a bacterially produced E7 fusion protein. Here we report that HPV16 E7 can interact with members of the AP1 family of transcription factors, including the c-Jun, JunB, JunD and c-Fos proteins. An E7 mutation which disrupts the formation of the zinc finger interfered with the ability of E7 to bind c-Jun, while a mutant E7 unable to bind pRb still displayed Jun binding activity. Using c-Jun deletion mutants, E7 binding localized to the region of c-Jun just upstream of and including the basic DNA binding domain, between amino acids 223 and 286. The ability of E7 to complex with c-Jun was confirmed *in vivo* using co-immunoprecipitation and a yeast two-hybrid system. Consideration of the data from the yeast system in conjunction with the *in vitro* binding data indicates that the c-Jun region involved in the E7 interaction lies between amino acids 224 and 249. When co-expressed with c-Jun in CV1P cells, E7 up-regulated the transcription from a single Jun-responsive promoter in transient assays, and a transformation-deficient c-Jun deletion mutation was shown to interfere with the transformation of REFs by E7 and ras.

Results

Interaction of E7 with cellular proteins

A glutathione-*S*-transferase (GST) fusion protein system was used to identify additional proteins that bind HPV16 E7. To confirm that the fusion protein system was capable of detecting genuine interactions with E7, the ability of the fusion protein to bind pRb from a cell lysate was tested and confirmed by Western blotting (data not shown).

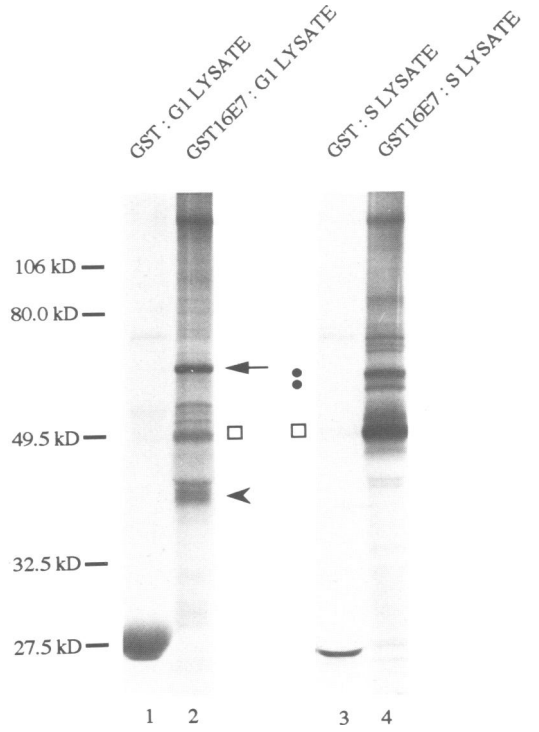

Fig. 1. Cell cycle-dependent interaction of A431 cellular proteins with GST16E7. Autoradiography of ^{35}S-labeled proteins which interact with GST alone (lanes 1 and 3) or GST16E7 (lanes 2 and 4). (←) The proteins bound by GST16E7 in G_1 phase lysates only. (●) S phase-specific bands. (□) A band observed in both extracts. The 39 kDa protein discussed in the text is indicated by an arrowhead.

To examine the possibility that E7 interacts with other cellular proteins, [^{35}S]cysteine- and methionine-labeled A431 cell lysates were prepared at different points in the cell cycle, cleared with GST protein alone to remove non-specific interactions and then incubated with GST16E7. A number of proteins were observed to associate with GST16E7 but not GST alone (Figure 1, compare lanes 1 with 2 and 3 with 4), and some of the associated proteins varied with the cell cycle (compare lane 2 with lane 4). Strong 39 (arrowhead) and 65 kDa (arrow) bands were present in the G_1 reaction but not in the S phase. Similarly, the S phase reactions exhibited two unique bands between 60 and 70 kDa (indicated by ●). The 60 kDa band may represent cyclin A, which has been previously described to associate with E7 (Tommasino *et al.*, 1993), but no further analysis of these proteins was undertaken. An unidentified band was also observed at 50 kDa in both phases of the cell cycle examined (□).

E7 binds to members of the AP1 family

The 39 kDa protein associated with E7 in G_1 was of particular interest. The band was not present in the S phase reactions, indicating that either the expression or the activity of this protein varied with the cell cycle. Such a pattern of expression, in combination with a previous report that the adenovirus E1A protein binds the 39 kDa c-Jun protein (Maguire *et al.*, 1991), led to the speculation that the 39 kDa band represented a member of the Jun family of transcription factors. This hypothesis was tested using an antibody which recognizes all three Jun isoforms to perform a Western blot of proteins interacting with

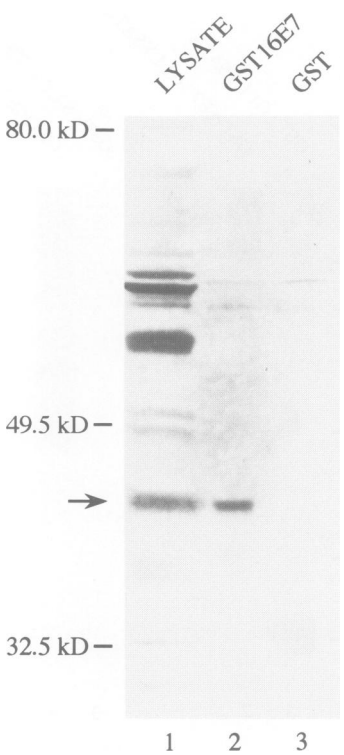

Fig. 2. Western blot analysis of keratinocyte cellular proteins using anti-c-Jun/AP-1 obtained from Oncogene Science (Uniondale, NY) which recognizes all three Jun isoforms. The 39 kDa Jun protein present in the lysate (lane 1) was also present in the proteins bound by GST16E7 (lane 2). GST alone showed no binding to Jun proteins (lane 3).

GST16E7 from G_1-synchronized secondary keratinocytes (Figure 2). The antibody recognized a 39 kDa protein in the GST16E7 reaction (lane 2) and the corresponding lysate (lane 1) but not in the GST reactions (lane 3), indicating that the 39 kDa protein interacting with E7 was a Jun-related protein.

Co-immunoprecipitation of c-Jun and E7

Immunoprecipitations using [^{35}S]cysteine- and methionine-labeled cell extracts were performed to determine if E7 and c-Jun were complexed in the HPV16 immortalized K1/16 keratinocyte line which expresses E7. As shown in Figure 3A, precipitation with the anti-Jun antibody brought down the ~39 kDa c-Jun protein in both K1/16 and the non-E7-expressing SCC-13 cell line (top panel). An ~19 kDa protein was also precipitated by the anti-Jun antibody in the K1/16 lysate but was not present in the SCC-13 anti-Jun precipitation (lower panel). This 19 kDa protein matched the E7 precipitated from K1/16 with the anti-E7 antibody and ran just slightly larger than the *in vitro*-produced E7 precipitated with the same antibody, possibly because of phosphorylation of the E7 at serines 31 and 32 (Barbosa *et al.*, 1990).

To confirm the specificity of the interaction, immunoprecipitations using anti-E7 and anti-c-Jun antibodies were carried out, followed by Western blotting with anti-E7 and anti-pRB antibodies (Figure 3B) on a cell line expressing HPV16 E7 alone. The anti-E7 antibodies precipitated E7 and pRB, a protein known to bind to E7 of

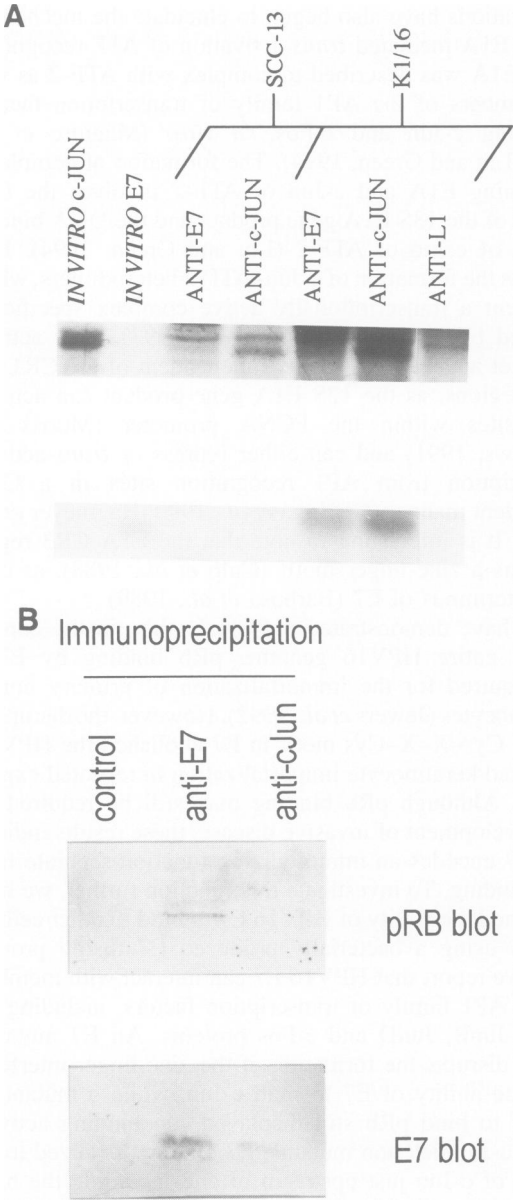

Fig. 3. (**A**) Co-immunoprecipitation of c-Jun and E7 from cells containing the whole HPV16 genome and expressing E7. R1066, an anti-E7 serum raised against an E7–β-galactosidase fusion protein, or 7064, an anti-c-Jun antiserum provided by Dr Edward Ziff, was used. A band representing E7 was precipitated by both anti-E7 and anti-c-Jun in the E7-expressing K1/16 cell line (lower panel) but was absent in precipitations from the HPV-negative SCC-13 cell line. (**B**) Co-immunoprecipitation of E7 from a cell line expressing HPV16 E7 alone (a gift from Dr J.K.McDougall) using anti-c-Jun antibodies, followed by Western blotting using alkaline phosphatase to detect E7 and pRB. E7 was co-immunoprecipitated with the anti-c-Jun antibody 7064 (lower panel). The monoclonal anti-E7 antibody (Santa Cruz Biotechnology Inc., CA), as expected, co-immunoprecipitated E7 (lower panel) and pRB (upper panel), while a non-specific antibody directed against the HPV16 major capsid protein, L1, precipitated neither protein.

HPV16. The anti-c-Jun antibodies also co-immunoprecipitated E7, while an unrelated control antibody precipitated neither protein. The c-Jun band immunoprecipitated by the E7 monoclonal antibody was obscured by the heavy chain from the antibody used to immunoprecipitate the E7–c-Jun complex, so the Western blot to detect c-Jun

Table I. Interaction of E7 and c-Jun detected using a yeast protein trap[a]

GAL4 TA fusion	GAL4 DB fusion	%GAL4 activity[b]
None	GAL4	100
E7	none	2
None	c-Jun	5
c-Fos	c-Jun	27
E7	c-June	32
None	c-Jun(Δ1–2149)	2
E7	c-Jun(Δ1–2149)	2

[a]Constructs cloned into pPC86, which encodes the GAL4 transactivation domain (TA), and pPC62, which encodes the GAL4 DNA binding domain (DB), are indicated in columns 1 and 2 respectively.
[b]Results are presented as the percentage of β-galactosidase activity detected when an intact Gal4 construct was transfected.

was difficult to interpret. Thus, c-Jun appears to co-immunoprecipitate E7 from two different cell lines expressing E7.

E7 interacts with c-Jun in a yeast two-hybrid system

To confirm that the E7–c-Jun interaction observed by co-immunoprecipitation was relevant, the yeast-based two-hybrid system was also used to detect the association between E7 and c-Jun *in vivo*. In this previously described system (Fields and Song, 1989), one protein of interest is cloned in-frame to the Gal4 DNA binding domain, while a second protein is fused to the Gal4 transcriptional activator domain. The fusion constructs are co-transfected into yeast, and interaction between the two proteins of interest reconstitutes an active Gal4 transcription factor. The level of β-galactosidase production from a Gal4-dependent reporter construct present in the yeast correlates with the strength of the interaction between the two proteins examined.

Table I shows the results of such an analysis performed using E7 and c-Jun fusions. E7 was fused to the Gal4 transcriptional activator, while c-Jun or c-Jun(Δ1–249), in which the N-terminus of c-Jun has been deleted up to amino acid 249, was fused to the DNA binding domain. As reported previously, when fused to the Gal4 DNA binding domain, the full-length c-Jun caused a low level of β-galactosidase production because of the presence of the c-Jun transcriptional activator domain (Chevray and Nathans, 1992). Transfection of the vectors with no insert, the E7-expressing vector alone or the c-Jun(Δ1–249) alone all gave background levels of β-galactosidase production. As expected, co-transfection of the c-Fos fusion and the c-Jun fusion together resulted in the production of β-galactosidase because of the leucine zipper-mediated dimerization of c-Jun and c-Fos. When E7 was co-expressed with c-Jun an even higher level of β-galactosidase production was observed, indicating a strong interaction between E7 and c-Jun. E7 did not appear to interact with c-Jun(Δ1–249), indicating that the region of contact may lie within the first 249 amino acids of c-Jun. This result provides further evidence that E7 and c-Jun interact in an *in vivo* situation.

Binding of E7 mutants to Jun proteins

To investigate further the interaction between the Jun proteins and E7, the ability of site-directed E7 mutants to associate with the Jun-related proteins was examined. The E7 mutants used here have been described previously (Edmonds and Vousden, 1989; Chesters *et al.*, 1990). Gly24 E7 has a mutation in the pRb binding domain that abrogates binding to pRb and rodent cell transformation, while Gly91E7 is a C-terminal mutant where the replacement of a cysteine in one Cys–X–X–Cys motif disrupts formation of the zinc finger. Gly91E7 retains its ability to bind pRb but has a greatly reduced rodent cell-transforming activity. The ability of these mutants to bind the Jun-related proteins is shown in Figure 4. *In vitro*-translated c-Jun bound to both GST16E7 and GSTgly24E7 (lanes 2 and 3) but failed to bind to GST and GSTgly91E7 (lanes 1 and 4), indicating that the zinc-finger domain of E7 is involved in the interaction with c-Jun but the pRb binding domain is not. However, because the rabbit reticulocyte lysates used to produce c-Jun contain high levels of pRb (unpublished data), it was possible that the association between E7 and c-Jun still required pRb. To test this possibility, c-Jun was produced in wheat germ extracts which are not known to contain any pRb homolog. GST16E7 was able to bind c-Jun in the absence of pRb, indicating that pRb is not required for the association of E7 and c-Jun (data not shown).

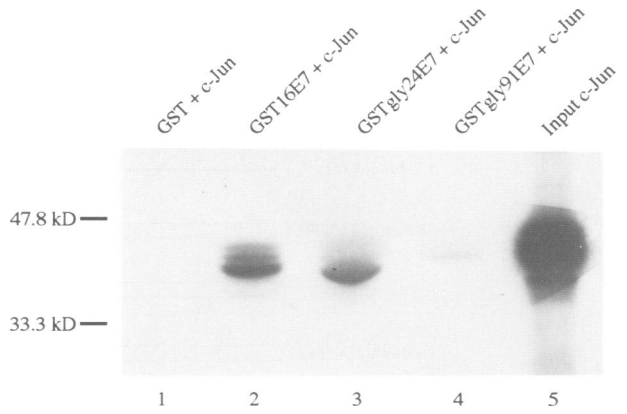

Fig. 4. Ability of site-directed E7 mutants to bind *in vitro*-produced c-Jun. [^{35}S]Methionine-labeled proteins were produced *in vitro* and tested for their ability to bind GST alone (lane 1), wild-type GST16E7 (lane 2) or GST16E7 point mutants (lanes 3 and 4). One half the amount of c-Jun used in the reactions is also shown (lane 5).

Identification of the c-Jun region involved in the interaction with E7

To define the region of c-Jun which interacts with E7, the previously described c-Jun deletion mutations (Alani *et al.*, 1991) shown in Figure 5A were produced *in vitro* and examined for their ability to bind GST16E7. Figure 5B shows that the deletion of amino acids 3–80 [c-Jun(Δ3–80), lane 7] and 3–122 [c-Jun(Δ3–122), lane 10] did not affect binding to GST16E7. Even mutant c-Jun(Δ3–223), which lacks the entire N-terminus of c-Jun, was still able to bind E7 (data not shown). The deletion of the C-terminus of the protein from amino acids 287–331 [c-Jun(Δ287–331), lane 8] also failed to disrupt complex formation, as did the insertion of four amino acids into the DNA binding domain (DBM-2, lane 9).

Further analyses using the c-Jun deletion mutations are shown in Figure 5C. Internal deletions of amino acids

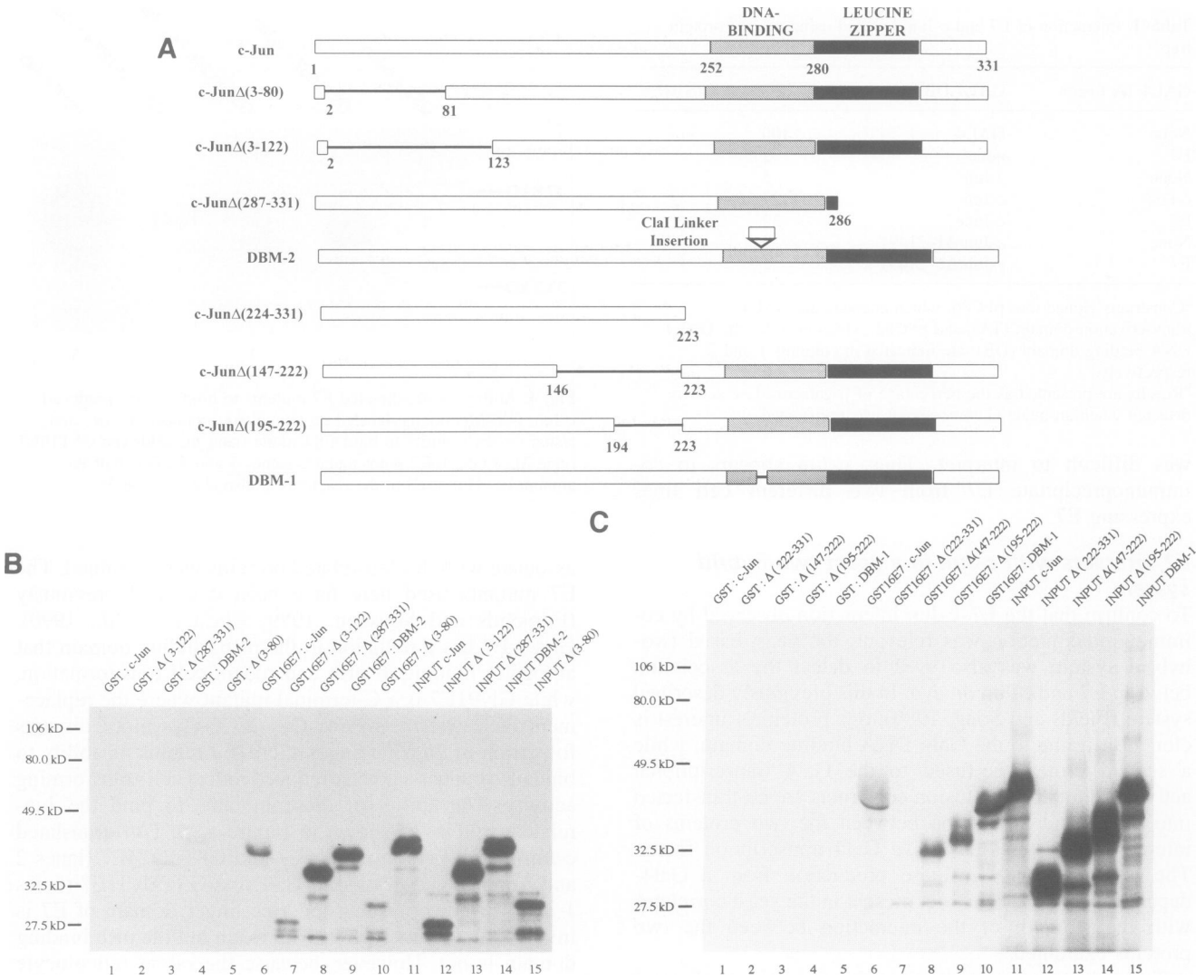

Fig. 5. Interaction of *in vitro*-produced c-Jun deletion mutants with GST16E7. (**A**) Schematic representation of the c-Jun deletion mutants used in this study. Lines represent the deleted region, while boxed areas represent the remaining sequences. (**B** and **C**) c-Jun deletions depicted in (A) were produced in a rabbit reticulocyte lysate transcription/translation system and mixed with either GST alone (lanes 1–5) or GST16E7 (lanes 6–10). One half the amount of input protein is shown for each deletion construct (lanes 11–15).

147–222 [c-Jun(Δ147–222), lane 8] or 195–222 [c-Jun-(Δ195-222), lane 9] did not interfere with the ability of c-Jun to bind E7. A two amino acid deletion within the basic DNA binding region, which abolishes the ability of Jun to bind DNA, did not alter the binding to E7 either (DBM-1, lane 10). However, deletion of the C-terminus of c-Jun from residues 224–331 completely abolished E7 binding activity [c-Jun(Δ224–331), lane 7]. Thus, the *in vitro* binding results indicate that the region of c-Jun which binds E7 lies between amino acids 224 and 286. The observation that c-Jun(Δ1–249) failed to bind E7 in the yeast two-hybrid system further delineates the binding region to amino acids 224–249, which lies just upstream of the DNA binding domain.

Identification of the Jun isoforms bound by E7

All analyses to this point had used c-Jun which had been produced *in vitro* or a pan anti-Jun antibody which recognizes all isoforms of the Jun proteins. Therefore, the ability of *in vitro*-produced JunB, JunD and c-Fos to bind to E7 was tested. As shown in Figure 6A, c-Jun, JunB and JunD were bound by the GST16E7 fusion protein (lanes 5–7), while GST alone bound none of the proteins (lanes 1–3). To control for non-specific charge interactions between the E7 zinc finger and basic DNA binding regions, a non-Jun-related bZIP transcription factor, VBP-αα (Iyer *et al.*, 1991), was *in vitro*-translated and tested for binding to GST16E7. As shown in Figure 6A, VBP-αα failed to bind GST16E7. Results obtained using *in vitro*-translated c-Fos are shown in Figure 6B. GST alone failed to bind, while GST16E7 strongly bound c-Fos. Because of previous reports that E1A binds to ATF-2 (Maguire *et al.*, 1991; Chatton *et al.*, 1993; Liu and Green, 1994), an ATF/CREB transcription factor which heterodimerizes with c-Jun (Hai and Curran, 1991), ATF-2, was produced *in vitro* and tested for binding to GST16E7; only a very weak interaction between E7 and ATF-2 was observed (data not shown). Hence, HPV16 E7 appears to bind all members of the AP1 family of transcription factors tested, including c-Jun, JunB, JunD and c-Fos.

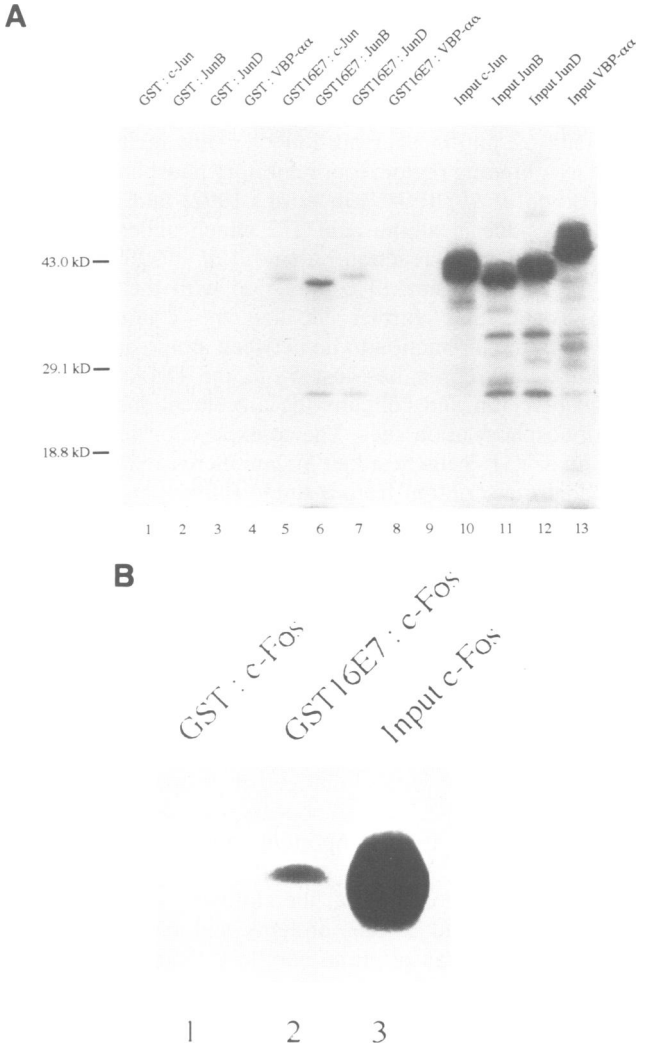

Fig. 6. Interaction of GST16E7 with *in vitro*-produced bZIP proteins. (**A**) c-Jun, JunB, JunD and VBP-αα were produced in a rabbit reticulocyte lysate transcription/translation system and mixed with either GST alone (lanes 1–4) or GST16E7 (lanes 5–8). One half the amount of input protein is shown for each protein (lanes 10–13). (**B**) c-Fos was produced *in vitro* and mixed with either GST alone (lane 1) or GST16E7 (lane 2). One half the amount of input protein is shown (lane 3).

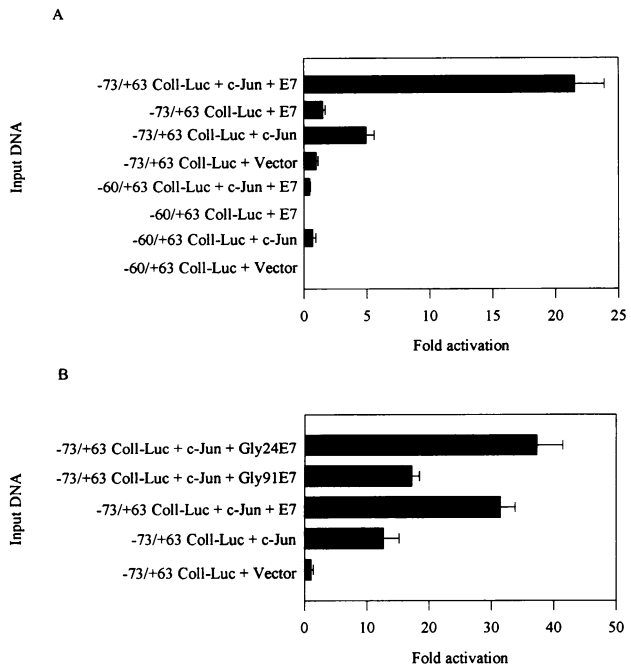

Fig. 7. Effect of E7 on c-Jun-regulated transcription. (**A**) E7 increases the response of a Jun-regulated promoter to c-Jun. CV1P cells were transfected with 1 μg of either –60/+63 Coll–Luc or –73/+63 Coll–Luc reporter and 1 μg each of the various expression vectors, as indicated and assayed for luciferase activity at 48 h post-transfection. Values indicated are the fold increase over –73/+63 Coll–Luc ± standard deviation. (**B**) Ability of E7 site-directed mutants to up-regulate the response of a Jun-regulated promoter to c-Jun. 1 μg of either wild-type E7 or mutated E7 was co-transfected as indicated with 1 μg c-Jun and 1 μg –73/+63 Coll–Luc reporter. Cells were harvested and lysates assayed for luciferase activity at 48 h post-transfection. Values indicated are the fold increase over –73/+63 Coll–Luc ± standard deviation.

Effect of E7 on c-Jun-induced transcription

Transient transfection assays were performed in CV1P cells to determine the effect of E7 on c-Jun-induced *trans*-activation. The results are shown in Figure 7A. Co-transfection of the RSV–c-Jun with the Jun-responsive –73/+63 Coll–Luc vector resulted in a 5-fold increase in luciferase activity compared with the vector control. *Trans*-activation by c-Jun increased to ~25-fold when the pKV-1 E7 expression vector was also transfected at a 1:1 ratio. No similar increase in activity was seen when the –60/+63 Coll–Luc reporter was examined, indicating that the *trans*-activation of c-Jun by E7 was specific for the single Jun-responsive element present in –73/+63 Coll–Luc. Hence, E7 is able to up-regulate c-Jun transcriptional activation activity from a c-Jun-responsive promoter in CV1P cells. Similar results were also seen using REFs and primary human keratinocytes (data not shown).

The E7 mutated proteins, which showed similar levels of expression when tested in NIH 3T3 cells (data not shown), were also tested for their ability to increase c-Jun-mediated *trans*-activation. As shown in Figure 7B, when co-transfected with c-Jun, Gly91E7 did not significantly *trans*-activate c-Jun-dependent transcription beyond that seen with c-Jun alone. Alternatively, Gly24E7, which is defective for pRb binding but can bind Jun proteins, retained a *trans*-activating activity equal to that seen with wild-type E7. Thus, the Jun-dependent *trans*-activating activity of the mutant E7 proteins correlates with Jun binding and not with the binding of pRb.

Effects of c-Jun expression on E7-transforming activity

As both E7 (Chesters *et al.*, 1990) and c-Jun (Schütte *et al.*, 1989) have been shown to cooperate with an activated *ras* gene in the transformation of REFs, it is possible that the disruption of Jun regulation could be required for E7-transforming activity. To test this possibility, c-Jun deletion mutations previously shown to be inactive for transformation (Alani *et al.*, 1991) were examined for their ability to the affect E7-transforming activity. As shown in Figure 8A, the co-transfection of c-Jun(Δ287–331), which has no transforming activity (Alani *et al.*, 1991) but retains the ability to bind E7 (see Figure 5B), reduced the number of transformed foci produced by c-Ha-*ras* and E7 by ~50%. Alternatively, the co-transfection of c-Jun(Δ224–331), which fails to bind

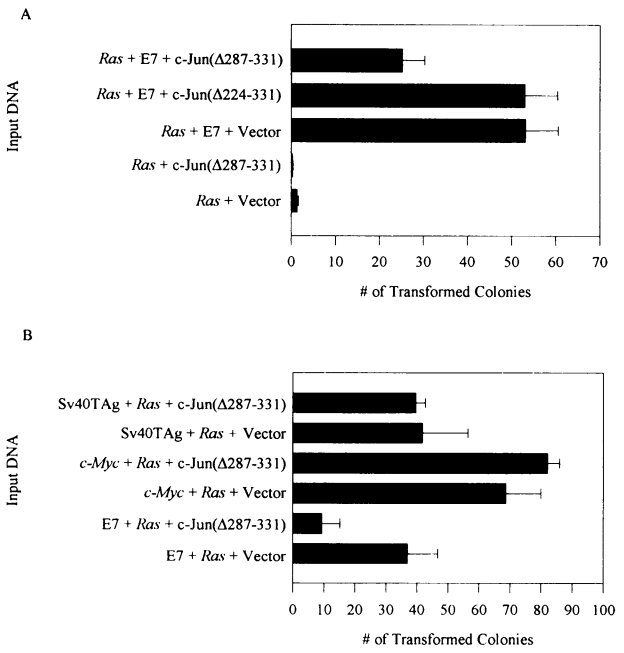

Fig. 8. (**A**) Reduction in REF transformation by E7 plus activated *ras* using a non-functional c-Jun construct which binds E7. c-Jun(Δ287–331) binds E7 but cannot dimerize or *trans*-activate, while c-Jun(Δ224–331) is also non-functional but does not bind E7. Cells were transfected with various expression vectors as indicated, and transformed foci were counted after 2 weeks in culture. Results were normalized for transfection efficiency and values indicated are the average number of transformed colonies ± standard deviation. (**B**) Ability of the c-Jun(Δ287–331) mutant to interfere with REF transformation by an activated *ras* plus an oncogene is specific for E7-induced transformation. E7, the SV40 T antigen or c-*Myc* were co-transfected with *ras* in the presence or absence of c-Jun(Δ287–331) and transformed foci were counted after 2 weeks in culture. Values indicated are the average number of transformed colonies ± standard deviation.

to E7 (see Figure 5C), did not affect the ability of E7 to cooperate with the activated *ras* gene in the transformation of REFs. This inhibition is specific for E7-induced transformation, as c-Jun(Δ287–331) had no effect on the ability of c-*Myc* or the SV40 large T antigen to transform REFs in cooperation with c-Ha-*ras* (Figure 8B). These data indicate that a c-Jun mutant, which can bind E7 but cannot *trans*-activate Jun-responsive transcription, can specifically interfere with the ability of E7 to cooperate with c-Ha-*ras* in REF transformation, possibly by sequestering E7 in an inactive complex. This observation indicates that Jun may be involved in the E7 transformation pathway and provides further evidence that the binding of E7 to Jun-related proteins is a physiologically relevant event.

Discussion

We have shown that, in addition to binding pRb, E7 is able to interact with AP1 transcription factors including c-Jun, JunB, JunD and c-Fos. The interaction with c-Jun has been shown to involve the zinc-finger domain of the E7 protein. The ability of a pRb binding-deficient E7 protein to bind c-Jun, and the observation that E7 can bind c-Jun produced in wheat germ extracts, indicate that the pRb itself is not required for the interaction between E7 and c-Jun. The ability of E7 to form complexes with c-Jun has also been confirmed by co-immunoprecipitation and a yeast-based two-hybrid system. Using c-Jun deletion mutations, we have also demonstrated that the region of c-Jun which binds E7 lies between amino acids 224 and 286. This 62 amino acid portion of c-Jun includes the basic DNA binding region, four inhibitory phosphorylation sites (Boyle *et al.*, 1991; Lin *et al.*, 1992) and a single cysteine residue at amino acid 272 which undergoes an inhibitory oxidation reaction (Abate *et al.*, 1990b). Data from the yeast system, in conjunction with the *in vitro* binding data, may further localize the c-Jun region involved in the interaction to lie between amino acids 224 and 249. This region lies just 5′ to the DNA binding domain of c-Jun and contains the aforementioned regulatory phosphorylation sites. The co-expression of E7 and c-Jun in CV1P cells resulted in an increase in c-Jun-dependent transcription from a single Jun-responsive element. A transcriptionally inactive c-Jun deletion mutant efficiently blocked the transformation of REFs by E7 and an activated *ras* gene, while a c-Jun deletion which could not bind E7 *in vitro* did not interfere with the transforming activity of E7. These results indicate that E7-mediated transformation might involve Jun interactions and that the E7–c-Jun interaction is biologically relevant to E7 function. This effect was specific for E7, as c-Jun(Δ287–331) had no effect on the transformation of REFs by c-*Myc* or the SV40 large T antigen in cooperation with an activated *ras* gene. Thus, it appears that the Jun family of proteins represents an important molecular target of HPV16 E7.

It is interesting to note that the ability of site-directed E7 mutants to bind to Jun proteins and *trans*-activate c-Jun-dependent transcription parallels their ability to immortalize primary keratinocytes in the context of the entire HPV16 genome (Jewers *et al.*, 1992). In that study, a HPV16 genome encoding the Gly24 site-directed E7 mutant was able to consistently give rise to immortalized keratinocyte cell lines, indicating that inactivation of the pRb is not a prerequisite for immortalization. The repeated failure of a Gly91E7-expressing genome to immortalize keratinocytes demonstrated that E7 is required for immortalization and may encode an activity separate from pRb binding. As Gly24E7 could both bind and *trans*-activate c-Jun, while Gly91E7 was defective for both binding and *trans*-activation, Jun binding by E7 may represent a necessary step in HPV-mediated immortalization. Experiments are currently underway to determine if the deregulation of Jun function can complement immortalization by the HPV16 genome encoding Gly91E7.

It is not surprising that the adenovirus E1A protein, which displays both structural and functional homology to E7 (Phelps *et al.*, 1988, 1991; Dyson *et al.*, 1992), has also been described to target the Jun proteins (Offringa *et al.*, 1990; Maguire *et al.*, 1991; Hagmeyer *et al.*, 1993; Liu and Green, 1994). Both proteins bind the retinoblastoma gene product and p107 via the conserved CR1 and CR2 domains (Whyte *et al.*, 1988; Dyson *et al.*, 1989; Davies *et al.*, 1993), and have been shown to activate transcription from the adenovirus E2 promoter via the E2F and ATF binding sites (Phelps *et al.*, 1991). It is known that E7 and E1A activate the E2F transcription factor by releasing E2F from transcriptionally inactive E2F–pRb and E2F–p107 complexes (Bagchi *et al.*, 1990;

Chellappan *et al.*, 1992). The mechanism by which E1A activates ATF binding sites is not as clearly understood, although E1A complexes with ATF-2 (Maguire *et al.*, 1991; Chatton *et al.*, 1993; Liu and Green, 1994) and can induce the formation of c-Jun–ATF-2 heterodimers (van Dam *et al.*, 1993). GST16E7 was also observed to form a complex with ATF-2, but the affinity of E7 for ATF-2 was much lower than that of E7 for the Jun proteins (data not shown). However, the CR3 region of E1A has also been shown to associate with c-Jun and c-Fos *in vitro* (Liu and Green, 1994), and E1A can either repress or *trans*-activate transcription from AP1 recognition sites in a CR1-dependent manner (Offringa *et al.*, 1990; Hagmeyer *et al.*, 1993). The zinc finger-dependent formation of complexes containing E7 and AP1 transcription factors, and the E7-mediated *trans*-activation of c-Jun described here, further demonstrate the conservation of function displayed between HPV16 E7 and the adenovirus E1A gene product.

Given the multiple regulatory motifs which co-localize with the E7 binding region of c-Jun, the binding of the Jun proteins by E7 could have a variety of possible outcomes. It is conceivable that the binding of Jun by E7 alters the tertiary structure of the Jun DNA binding region. Such an alteration could affect Jun DNA binding activity or could target Jun to a different DNA recognition sequence. c-Jun activity is also regulated by phosphorylation, and the region of c-Jun implicated in complex formation with E7 contains multiple important regulatory phosphorylation sites. The amino acids important in this regulation lie just upstream of the DNA binding domain, and include serines at positions 243 and 249 and threonines at positions 231 and 239 (Boyle *et al.*, 1991). It has been shown that *in vitro* phosphorylation of three of these sites (Thr239, Ser243 and Ser249) by glycogen synthetase kinase 3 (GSK3) inhibits c-Jun DNA binding (Boyle *et al.*, 1991). The phosphorylation of threonine 231 and serine 249 by CKII *in vitro* has also been shown to inhibit c-Jun DNA binding activity, and these sites, as well as serine 243, have been demonstrated to be phosphorylated on the inactive form of c-Jun found in quiescent cells (Lin *et al.*, 1992). Because CKII is a predominantly nuclear protein (Krek *et al.*, 1992) and the inhibition of CKII *in vivo* increases AP1 transcriptional activity (Lin *et al.*, 1992), it is believed that CKII may be a major regulator of c-Jun activity *in vivo*. It is important to note that HPV16 E7 has also been shown to be phosphorylated by CKII on two serine residues (Firzlaff *et al.*, 1989). The phosphorylation of both c-Jun and E7 by CKII may be important for a variety of reasons. First, the phosphorylation events could coordinate c-Jun and E7 activity through the cell cycle. Second, the co-localization of c-Jun and CKII by E7 could increase or interfere with the inhibitory phosphorylation of c-Jun. Third, E7 may affect the ability of CKII to phosphorylate c-Jun by masking or making available the amino acids actually contacted by CKII. Further studies which examine the phosphorylation state of c-Jun in E7-expressing cells are being undertaken to address this issue.

Another possibility is that E7 affects the redox regulation of c-Jun activity. c-Jun contains a single cysteine residue within the DNA binding domain, at amino acid 272, which has been reported to undergo oxidation *in vitro* (Abate *et al.*, 1990b). This oxidation inhibits the c-Jun DNA binding activity and can be reversed by the ubiquitous Ref-1 cellular enzyme or treatment with thiol compounds (Abate *et al.*, 1990a; Xanthoudakis and Curran, 1992). Two lines of evidence indicate that the redox regulation of c-Jun activity is not the target of E7 binding. First, the omission of DTT in *in vitro* binding reactions did not affect the ability of GST16E7 to complex with c-Jun (data not shown). Second, GST16E7 was able to bind the DBM-1 mutant (Figure 5C, lane 10), a deletion mutant which does not contain cysteine 272 and lacks any DNA binding activity.

It is also possible that E7 may alter the ability of c-Jun to form complexes with the many cellular proteins which are known to heterodimerize with c-Jun, as has been demonstrated for E1A (van Dam *et al.*, 1993). c-Jun has been shown to dimerize with the Fos proteins, including c-Fos, FosB, Fra-1 and Fra-2 (Halazonetis *et al.*, 1988; Cohen *et al.*, 1989; Zerial *et al.*, 1989; Kovary and Bravo, 1992), members of the ATF family (Hai and Curran, 1991), NF–IL6 (Hsu *et al.*, 1994), the Maf nuclear oncoprotein (Kataoka *et al.*, 1994) and MyoD (Bengal *et al.*, 1992). These various heterodimeric complexes have different optimal DNA recognition sequences, creating a complex transcriptional regulation mechanism. By altering the affinity of c-Jun for any of these factors, E7 could activate the transcription of genes containing specific promoter sequences. Such a mechanism is especially attractive for E7, because E7 has been demonstrated to increase transcription from ATF binding sites (Phelps *et al.*, 1988, 1991); indeed we observe weak complex formation between E7 and ATF-2. Further studies will be required to determine if E7 is capable of altering the formation of heterodimers by Jun-related proteins.

In summary, we have shown that (i) E7 binds to members of the AP1 family of transcription factors, (ii) E7 can increase the response of a Jun-dependent promoter to c-Jun, and (iii) a c-Jun deletion mutation which can bind E7 interferes with the ability of E7 and an activated *ras* to cooperate in REF transformation assays. The interaction of E7 with AP1 factors may be important in the viral life cycle for a variety of reasons. First, the transcription of the E6 and E7 genes from the HPV16 viral promoter is controlled in part by the AP1 sites present in the promoter (Chan *et al.*, 1990). Because this same promoter region also acts as the origin of replication, it is possible that the E7–Jun complexes play a role in the control of viral replication. It has been demonstrated previously that cellular transcription factors can play a role in the replication of DNA viruses (Gou and DePamphilis, 1992). Work in our laboratory has shown that an AP1 binding site can contribute *in cis* in transient HPV11 replication assays (Y.Sun and D.J.McCance, unpublished observations). Alternatively, an attractive hypothesis is that through this interaction, E7 interferes with the AP1 regulatory activity during the differentiation of keratinocytes. AP1 transcription factors have been described to play an important regulatory role in the differentiation of keratinocytes (Jehn *et al.*, 1992), and the HPV life cycle is closely tied to the differentiation state of the host keratinocyte cells. Viral replication relies on host DNA replication enzymes and nucleotide pools which would normally be absent in the terminally differentiated epidermal cells where HPV

replication occurs. The binding of Jun proteins by E7 may result in an inhibition of the differentiation required for efficient virus replication. In some cases, the ultimate outcome of inhibiting keratinocyte differentiation could be the development of the premalignant or malignant epithelial cell disease which has been associated with HPV16. Further analyses of how E7 disrupts normal c-Jun regulation will aid in the understanding of the association between HPV infection and the development of malignant disease.

Materials and methods

Cells and cell culture

Primary keratinocytes were isolated from fresh foreskin pieces and maintained in KGM medium (Clonetics, San Diego, CA). A431 adenocarcinoma cells, C33A cervical carcinoma cells and CV1P African green monkey kidney cells were all grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS). REFs were isolated as described previously (Birrer *et al.*, 1991) and maintained in DMEM with 10% FBS. SCC-13, a squamous cell carcinoma cell line, and K1/16, an HPV16-immortalized keratinocyte line, were maintained in E medium (Wu *et al.*, 1982) with mytomycin C-treated J2 3T3 mouse fibroblast feeder cells.

Recombinant DNA

Wild-type E7 expressed in pUC19.MoLTR (pKV1) and E7 containing point mutations at amino acids 24 (Gly24E7) and 91 (Gly91E7) were provided by Dr Karen Vousden (Edmonds and Vousden, 1989). pGEX16E7 contains the HPV16 E7 reading frame cloned in-frame with the GST gene in the pGEX2T expression plasmid (Pharmacia, Piscataway, NJ). The mutant E7 genes were PCR-amplified and the PCR products were cloned into the PCR II vector provided with the TA Cloning Kit (Invitrogen, San Diego, CA) following the manufacturer's instructions. E7 inserts were removed from PCR II by *Bam*HI–*Eco*RI digestion, purified using Magic PCR Preps (Promega, Madison, WI) and ligated into *Bam*HI–*Eco*RI linearized pGEX2T. The resulting clones were sequenced to confirm the presence of the desired mutations.

Plasmids –73/+63 Coll–CAT and –60/+63 Coll–CAT were provided by Dr Michael Karin and used to clone the respective fragments of the collagenase promoter into the pXP1 luciferase reporter construct. The –73/+63 Coll fragment, which contains one AP1-responsive element, was removed by *Hin*dIII–*Kpn*I digestion and ligated into pXP1 to yield –73/+63 Coll–Luc. The –60/+63 collagenase promoter fragment was PCR amplified using primers which introduced a *Bam*HI at the 5′ end of the insert. The PCR product was removed from the TA vector by *Bam*HI/*Xho*I digestion and cloned into *Bam*HI/*Xho*I linearized pXP1 using the cloning methods described above, to yield the –60/+63 Coll–Luc vector. Activated c-Ha-*ras* (pEJ 6.6) was a gift from R.Weinberg.

T7Fos and T7JunB were provided by Dr Michael Karin (Deng and Karin, 1993), while T7JunD was provided by Dr Moshe Yaniv (Hirai *et al.*, 1989). pRcRSV c-Jun was provided by Dr Ian Morgan and was used to clone the c-*jun* gene into *Sma*I–*Hin*dIII-linearized pGEM2 (Promega) to allow T7-driven transcription. All c-Jun deletion mutants (Figure 5) have been described previously (Alani *et al.*, 1991). T7ATF-2 was provided by Dr Michael Green (Kim *et al.*, 1992), while T7 VBP-αα was provided by Dr John Burch (Iyer *et al.*, 1991).

Fusion protein expression and purification

GST fusion proteins were expressed and purified as described previously (Smith and Johnson, 1988), with some modification. After reaching log phase at 37°C, cultures were cooled to room temperature, IPTG-induced and shaken overnight at room temperature because the induction of cultures at 37°C yielded insoluble GST16E7. After purification, proteins were analyzed by SDS–PAGE and Coomassie staining to determine the purity and quantity of protein produced. Preparations were typically >90% pure fusion protein.

Cell lysate preparation

To synchronize cells in G_0, 70–80% confluent 100 mm plates of cells were washed with PBS and incubated in cysteine and methionine-free DMEM containing 4% dialyzed FCS for 2 days. To obtain G_1 phase lysates, the synchronized cells were labeled in cysteine- and methionine-free DMEM with 10% FCS and 0.5 mCi [^{35}S]Express cysteine and methionine for 3 h. Keratinocyte medium was supplemented with 5 ng/ml epidermal growth factor during labeling. Cells were washed three times with PBS and incubated in 1 ml EBC [50 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml pepstatin] for 30 min on ice. Cells were scraped into microcentrifuge tubes and cellular debris was pelleted. To obtain S phase cultures, G_0-synchronized cells were incubated in DMEM with 10% FCS and 0.5 mM hydroxyurea. After 20 h, cells were washed extensively to remove the hydroxyurea and incubated for 5 h in cysteine- and methionine-free DMEM with 10% FCS and 0.5 mCi [^{35}S]Express cysteine and methionine, prior to harvesting as described above. For Western blots, G_0-synchronized cells were re-stimulated in DMEM with 10% FCS or E medium for keratinocytes and harvested as described above.

Detection and identification of proteins interacting with E7

To detect cellular proteins that interact with E7, one-tenth of a 100 mm plate lysate prepared above was first cleared with ~5 μg GST alone bound to the Sepharose beads. After clearing for 3 h with rotating at 4°C, the beads were pelleted and the lysate was added to ~5 μg the appropriate fusion protein. Following a 2 h incubation with rotating at 4°C, the beads were centrifuged and washed five times in EBC prior to analysis by SDS–PAGE. Proteins were visualized by autoradiography. For comparison, proteins bound by GST alone were also analyzed in all assays described. For Western blots, unlabeled lysates were incubated with the fusion proteins as described above, but the bound proteins were transferred to nitrocellulose after electrophoresis. Membranes were probed with antibodies according to the antibody manufacturer's recommendations. Western blots were visualized using an alkaline phosphatase development kit (Bio-Rad, Melville, NY).

E7 interactions with in vitro-produced proteins

All *in vitro* reactions were performed using [^{35}S]methionine in TNT-coupled transcription/translation systems (Promega) according to the manufacturer's instructions. 50 000 c.p.m. protein were diluted in 150 μl application buffer [20 mM HEPES (pH 7.9), 80 mM KCl, 6 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1 mM DTT, 1 mm ATP], cleared with GST alone and then incubated with ~2 μg fusion protein attached to agarose beads with rotating at 4°C for 2 h. The beads were washed five times with application buffer and bound proteins were analyzed by SDS–PAGE and autoradiography.

Immunoprecipitations

^{35}S-labeled lysates were prepared from G_1-synchronized cells as described above but ELB$^+$ (250 mM NaCl, 0.1% NP-40, 50 mM HEPES, 1 mM PMSF, 5 mM EDTA) was used in place of EBC. One half of the lysate from one 100 mm plate was used for each reaction. Lysates were cleared with a non-specific antibody and protein A–Sepharose beads at 4°C for 2 h before beads were pelleted and the supernatant was used for immunoprecipitation with 40 μl specific antiserum. Reactions were rotated overnight at 4°C, protein A–Sepharose beads were added for 1 h, and bound complexes were pelleted and washed five times with ELB$^+$. Proteins were resolved by SDS–PAGE and visualized by autoradiography. For the monoclonal E7 antibody, a secondary rabbit anti-mouse antibody was added for 2 h prior to the addition of protein A–Sepharose beads. The 7064 and the anti-E7 antibodies were used at 1:3000 and 1:200 dilution respectively for the Western blots.

Yeast two-hybrid system

The yeast two-hybrid system used in these studies has been described previously (Fields and Song, 1989; Chevray and Nathans, 1992). The yeast strain used was SFY526 (*MAT*α, *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3*, *112*, *can*^{r}, *gal4-542*, *gal 80-538*, URA3::GAL1–lacZ; Bartel *et al.*, 1993). This strain is deleted for GAL4 and GAL80 and contains an integrated GAL1–lacZ activated by UAS_G. Yeast was grown in YEPD or selective minimal medium (Ausubel *et al.*, 1990) and transformations were performed by electroporation (Guthrie and Fink, 1991). β-Galactosidase activity was assayed on plates and quantified in liquid (Ausubel *et al.*, 1990).

Construction of GAL4 fusion vectors

Plasmid vectors pPC62 [GAL4 DNA binding domain (DB)] and pPC86 [GAL4 *trans*-activation domain (TA)] were obtained from Dr Daniel Nathans, as were the fusion vectors bz-Jun(TA), c-Jun(TA), bz-Jun(DB), c-Fos(TA) and pPC2, which encodes full-length GAL4 (Chevray and

Nathans, 1992). The full-length E7 gene was inserted into the GAL4(TA) vector pPC86 at the *Eco*RI and *Spe*I sites.

Transient transfection assays

The effect of E7 on c-Jun-induced transcription was studied in CV1P cells using lipofectamine (Gibco-BRL) as per the manufacturer's instructions. Briefly, 90% confluent plates were split at 1:8 onto 100 mm tissue culture dishes 24 h prior to transfection. Cells were washed and a total of 3.5 μg DNA and 13 μl lipofectamine in 2.5 ml DMEM was added to each plate. After 6 h, 3 ml DMEM plus 10% FCS was added to the transfection mix. After 20 h, plates were washed with PBS and incubated in DMEM with 10% FCS for another 24 h. Plates were transfected in groups of four, with one plate being stained *in situ* for β-galactosidase activity to control for transfection efficiency and three being harvested and assayed for luciferase activity. All results are expressed as the fold increase over the activity of −73/+63 Coll–Luc ± standard deviation and represent at least two independent experiments.

Co-transformation assay

The inhibition of E7-induced transformation of REFs by c-Jun(Δ287–331) was measured using a co-transformation assay in which primary REFs were transformed by the transfection of an activated c-Ha-*ras* gene in combination with E7 and the various c-Jun deletion constructs. The cells were transfected with 10 μg c-Ha-*ras* gene, 10 μg pKV-1 E7 expression vector, 10 μg c-Jun deletion construct and 5 μg pRC, which carries the neomycin resistance gene. The cells were washed the following day and transformed foci were counted after 2 weeks in culture. Results were normalized for transfection efficiency, as determined by the number of G418-resistant colonies. All transfections were performed four times in triplicate and the results are expressed as the mean number of transformed foci ± standard deviation.

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