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Characterization of the human papillomavirus E2 protein: evidence of *trans*-activation and *trans*-repression in cervical keratinocytes

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The major regulator of papillomavirus transcription is encoded by the viral E2 gene. The E2 gene has been well characterized in bovine papillomavirus (BPV) where it encodes at least three different polypeptides which differentially affect viral gene expression. In human papillomaviruses (HPVs) the E2 gene product is much less well characterized. In this study we have analysed the mechanism of action of the HPV-16, HPV-18 and BPV-1 E2 proteins in cervical keratinocytes. We show that the full length HPV E2 protein acts as a potent transcriptional activator of viral gene expression in both normal and immortalized keratinocytes. In contrast, the BPV-1 E2 protein produces transcriptional repression under identical conditions. A cDNA encoding the C-terminal half of the HPV-16 E2 protein in these assays weakly repressed viral gene expression. Further, co-transfection of this cDNA with the full length clone progressively abolishes the activation *in trans* by the full length HPV E2 protein. Gel retardation assays have defined a number of protein complexes between the long and short forms of E2 but with no evidence for preferential DNA binding. These results define two distinct activities for the HPV-16 E2 protein, indicate functional differences with the BPV E2 protein and suggest that splicing of the HPV E2 mRNA is a critical mechanism for controlling viral gene expression.

Key words: E2 gene/keratinocytes/papillomavirus/transcription

Introduction

Human papillomaviruses (HPVs) have been closely linked to the development of cervical cancer (zur Hausen and Schneider, 1987). Studies *in vitro* have shown the virus to be capable of immortalizing both rodent cells and human keratinocytes (Dürst *et al.*, 1987; Matlashewski *et al.*, 1987; Pirisi *et al.*, 1987; Storey *et al.*, 1988), the latter being the natural target cells of the virus *in vivo*. Immortalization of human cervical keratinocytes by HPV is primarily dependent upon the functional viral E6 and E7 gene products (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989). Recently however, we identified a variant HPV-16 which failed to induce immortalization of human cervical keratinocytes when the viral genome was under the control of its homologous promoter (Storey *et al.*, 1992). This virus was shown to contain a mutation in the E2 open reading frame which resulted in a premature stop codon. Co-transfection experiments with a wild-type E2 restored the ability of this virus to induce immortalization of human keratinocytes. These experiments suggested that the E2 protein functioned in a positive fashion in the immortalization process, possibly as an activator of viral gene expression. This conclusion was supported by previous studies which showed that addition of exogenous E2 protein could increase the ability of the virus to cooperate with an activated *ras* oncogene to transform primary rodent cells (Lees *et al.*, 1990). Together with transient transfection studies which showed that HPV-16 E2 could *trans*-activate the HPV-16 upstream regulatory region (URR) in established cell lines (Cripe *et al.*, 1987; Phelps and Howley, 1987), these results suggested that E2 was up-regulating the level of expression of the viral oncoproteins E6 and E7, in cervical keratinocytes. However, in a separate series of experiments it was reported that the full length HPV-18 E2 and the BPV-1 E2 proteins functioned as repressors of viral gene transcription in normal keratinocytes (Thierry and Yaniv, 1987; Bernard *et al.*, 1989). This led to the hypothesis that deletion of the E2 open reading frame (ORF), which is often observed in late stage tumours and cell lines (Schwarz *et al.*, 1985; Baker *et al.*, 1987), is an integral part of the immortalization process *in vivo*, a consequence of which would be deregulation of viral E6 and E7 gene expression. Recent immortalization studies showed that HPV-16 mutated in the E2 ORF gave rise to a higher efficiency of immortalization, thus tending to support this hypothesis (Romanczuk and Howley, 1992). However, mutants in the E1 ORF and the E2 DNA binding sites within the viral URR also showed increased frequency of immortalization, indicating that the effect was not solely due to transcriptional regulation by the HPV-16 E2 protein (Romanczuk and Howley, 1992). In addition, recent studies have also shown that disruption of E2 is often a late event in tumour formation, since intact E2 has been reported in a number of pre-malignant lesions (Matsukura *et al.*, 1989; Dürst *et al.*, 1992).

In this report we describe a series of experiments designed to elucidate the transcriptional activity of the HPV-16, HPV-18 and BPV-1 E2 proteins in human keratinocytes. We show that the HPV E2 protein functions as a transcriptional activator of the HPV-16 and HPV-18 promoters in both normal and immortalized cervical keratinocytes whereas the BPV E2 protein functions as a transcriptional repressor. A cDNA encoding sequence derived from the E1 region and the C-terminal domain of the HPV-16 E2 gene (Doorbar *et al.*, 1990), analogous to one of the short repressor forms of BPV-1 E2 (Lambert

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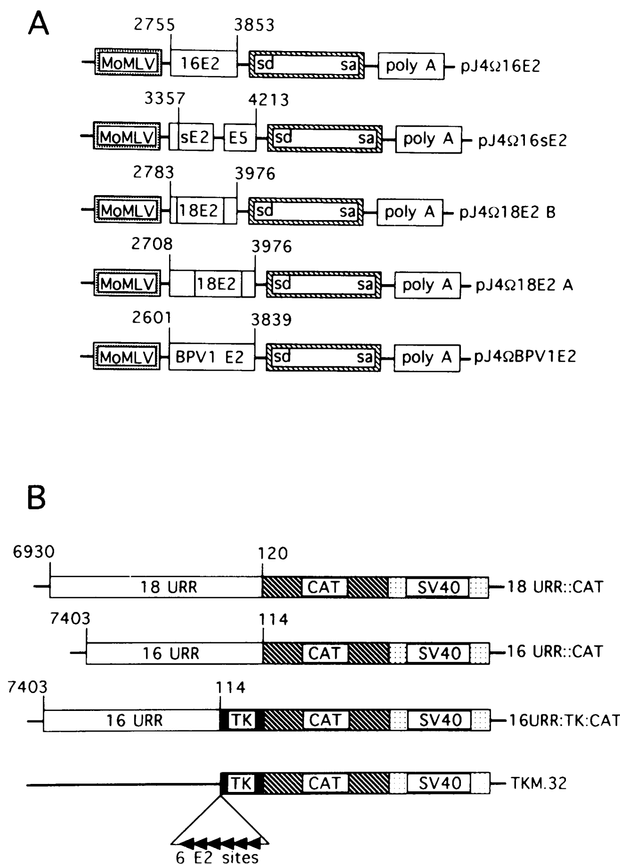

Fig. 1. (**A**) Papillomavirus E2 expression plasmids and (**B**) CAT constructs, used in this study. Construction of the plasmids is described in Materials and methods. Numbers correspond to nucleotide numbers on the HPV-16, HPV-18 and BPV-1 genomes. Also shown is the Moloney murine leukaemia virus (MoMLV) LTR and SV40 splice donor (sd), splice acceptor (sa) and polyadenylation sequences (poly A). The diagram is not to scale.

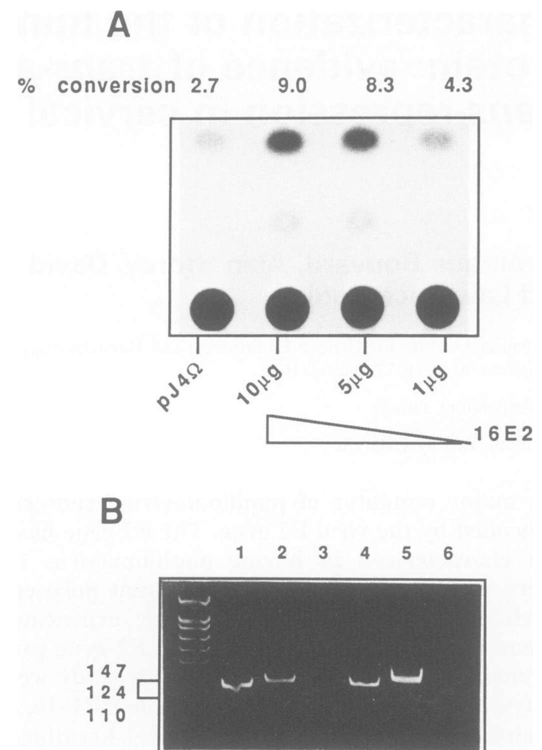

Fig. 2. (**A**) Activation of the HPV-16 URR by pJ4Ω16E2. The HPV-16 URR::CAT plasmid was transfected into cells together with either 10 μg of pJ4Ω or the indicated amounts of pJ4Ω16E2. After 48 h cells were harvested and CAT activity measured. Numbers show percentage CAT conversion. Cells used are normal primary cervical keratinocytes at passage 2. (**B**) HPV-16 transcription originating from the region located between positions 80 and 97 on the URR. Total RNA extracted from human keratinocytes transfected with 16URR::CAT and pJ4Ω (lanes 1, 2 and 3) or pJ4Ω16E2 (lanes 4, 5 and 6) was reverse transcribed using a primer lying at the start of the CAT gene. cDNAs were then PCR amplified using the above primer and one of the three primers lying on the 16 URR at positions 97–114 (lanes 1 and 4), 80–96 (lanes 2 and 5) and 62–79 (lanes 3 and 6). PCR products were resolved by PAGE. Molecular weight markers are 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110 and 67 bp.

et al., 1987; Choe *et al.*, 1989; Vaillancourt *et al.*, 1990), was also analysed and shown to weakly repress the basal level of transcription. Furthermore we demonstrate that this shortened protein abolishes the activity of the full length HPV E2 protein. Gel retardation analyses indicate that repression may be through the formation of heteromeric forms of the HPV E2 protein.

Results

HPV-16 E2 activates the HPV-16 promoter in cervical keratinocytes

In order to investigate the activity of the viral E2 gene products on viral transcription, a series of E2 expression plasmids and CAT reporter plasmids were constructed (Figure 1). In the first series of experiments we were interested in determining the activity of the HPV-16 E2 protein upon the HPV-16 promoter. The full length E2 expression plasmid, pJ4Ω16E2 (Figure 1), was co-transfected with the HPV-16 URR::CAT reporter plasmid into human keratinocytes. Cells were harvested after 48 h and the results obtained are shown in Figure 2. From this analysis it is clear that the pJ4Ω16E2 construct activates expression of the HPV-16 URR in human keratinocytes in a dose dependent manner. At this stage it was necessary to demonstrate that the HPV-16 p97 promoter was being utilized. In order to do this, cells were transfected as above and RNA was isolated after 48 h. Using a primer lying within the beginning of the CAT gene, the RNA was then reverse transcribed and cDNA amplified by polymerase chain reaction (PCR) with one of three additional primers. These 18mer oligonucleotides are located at positions 62–79, 80–96 and 97–114 on the HPV-16 DNA; the results obtained are shown in Figure 2B. Only primers 80–96 and 97–114 gave PCR products of the predicted size, 135 and 118 bp respectively. This confirmed that the HPV-16 transcripts originated in the vicinity of the p97 promoter and is in agreement with previous published observations (Smotkin and Wettstein, 1986).

Transcriptional activation by HPV-18 E2

Previous studies which have reported transcriptional repression by HPV E2 have been done using the HPV-18 E2 protein. It was possible that the differences we observed with HPV-16 E2 were due to the two proteins not being functionally equivalent. To test this hypothesis we cloned

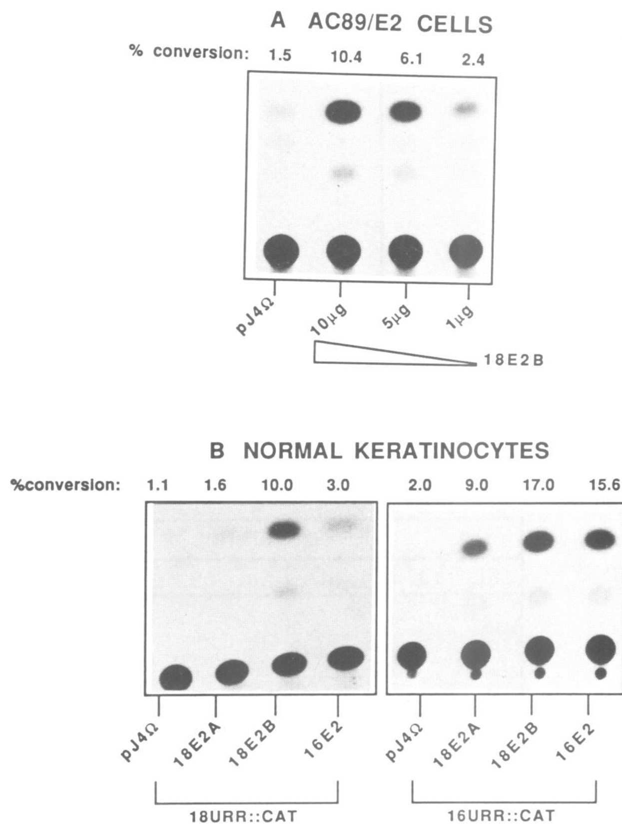

Fig. 3. Comparison of HPV-16 and HPV-18 E2 activation of different HPV URRs. (**A**) Titration of pJ4Ω18E2B. Indicated amounts of HPV-18 E2 expression plasmid were co-transfected with the 18URR::CAT plasmid. Assays were performed 48 h post-transfection. Numbers show percentage CAT conversion. (**B**) 7 μg of each URR CAT plasmid were cotransfected with 5 μg of either pJ4Ω, pJ4Ω16E2, pJ4Ω18E2A or pJ4Ω18E2B as indicated into normal cervical keratinocytes at passage 2. Numbers show percentage CAT conversion.

the HPV-18 promoter into pBLCAT3 (HPV-18 URR::CAT construct; Figure 1) and also produced two clones of the HPV-18 E2 gene in pJ4Ω. One corresponds to the E2 construct described previously (Bernard *et al.*, 1989) and is termed pJ4Ω18E2A, the second has a much reduced length of 5′ non-coding sequence prior to the initiation codon and is termed pJ4Ω18E2B (Figure 1). These E2 expression plasmids were co-transfected with the HPV-18 URR::CAT plasmid into human keratinocytes, along with pJ4Ω16E2 for comparison. The results obtained are shown in Figure 3 and demonstrate that pJ4Ω18E2B indeed activates the level of transcription from the HPV-18 URR::CAT reporter plasmid in a dose dependent manner (Figure 3A) similarly to HPV-16 E2. Comparison of the different E2 expression plasmids in Figure 3B reveals two interesting points. First, HPV-16 and HPV-18 URRs are activated by both the homologous and heterologous E2 proteins, thus indicating a conservation of function between the two viruses. Secondly, in comparison with pJ4Ω18E2B, pJ4Ω18E2A produces only weak activation of the HPV-18 promoter. It is possible that the long leader sequence which contains both in- and out-of-frame ATGs upstream of the E2 start codon reduces the expression of E2. To address this possibility we compared the ability of the two HPV-18E2 constructs to activate expression from an artificial promoter containing 6× E2 binding sites and

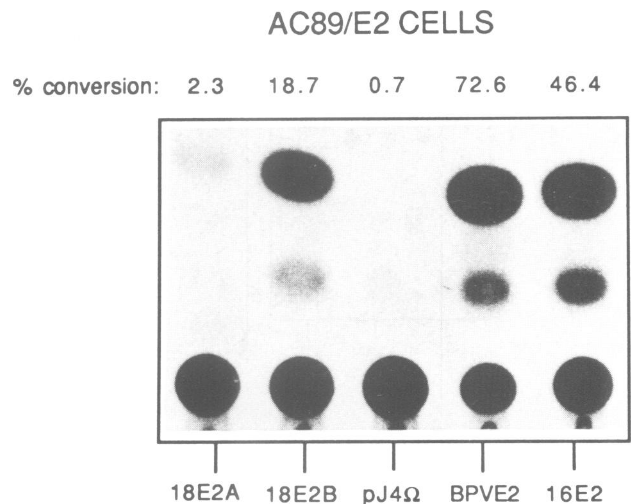

Fig. 4. Comparison of the ability of different E2 expression plasmids to activate expression from the artificial promoter CAT plasmid, TKM:32. 2 μg of TKM:32 were transfected together with 10 μg of the indicated plasmid into AC89/E2 cells. Cells were harvested after 48 h and CAT activity measured. Numbers show percentage CAT conversion.

the TK promoter upstream of the CAT gene. This plasmid, TKM:32, was kindly provided by M.Yaniv and F.Thierry, and has been used previously to assess levels of E2 expression (Thierry *et al.*, 1990). The results obtained in Figure 4 demonstrate that pJ4Ω18E2B gave a much higher activation than pJ4Ω18E2A. Since the E2 proteins encoded by the two plasmids are identical, these results support our conclusion that pJ4Ω18E2B produces higher levels of E2 expression than pJ4Ω18E2A. Taken together, these results also demonstrate that HPV-18 E2 functions as a transcriptional activator in normal cervical keratinocytes.

Transcriptional repression of the HPV URR by BPV-1 E2

Previous studies have often used BPV-1 E2 as a model for the transcriptional activity of HPV E2. This is not surprising since the proteins share some degree of homology in primary amino acid sequence, bind to the same recognition sequence and activate heterologous promoters containing E2 recognition sequences. However, BPV-1 E2 strongly represses the HPV URR and this fact has been used to propose a function for HPV E2. We were therefore interested in comparing the activity of the BPV-1 E2 and HPV-16 E2 proteins in our transcriptional assays. Keratinocytes were transfected as above with pJ4ΩBPV1E2 and pJ4Ω16E2 together with one of two CAT reporter plasmids. The results obtained are shown in Figure 5. It is clear that under conditions where the BPV E2 expression plasmid induced a dramatic repression of HPV promoter activity the HPV-16 E2 plasmid strongly activated viral gene expression (Figure 5A). As a control for this experiment, cells were transfected with either HPV-16 E2 or BPV-1 E2 plus HPV-16 URR:TK:CAT. In this case the TK promoter is used and both HPV-16 and BPV-1 E2 proteins produced strong *trans*-activation. Similar results were also obtained when the TKM:32 reporter plasmid was used (Figure 4). In this case, at equivalent input levels of BPV-1 and HPV-16 E2 expression plasmids, BPV-1 E2 gave an ~1.5-fold higher level

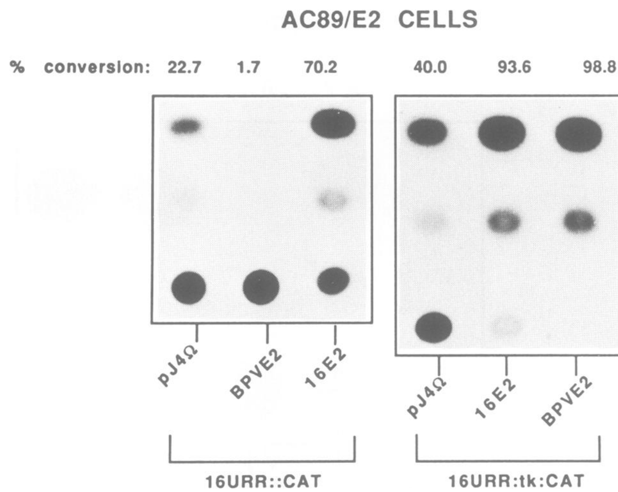

Fig. 5. Comparison of the transcriptional activity of the HPV-16 E2 and BPV-1 E2 proteins. 7 μg of 16URR::CAT or 5 μg of 16URR:TK:CAT were transfected together with 5 μg of the indicated plasmid. Cells were harvested after 48 h and CAT activity measured. Numbers show percentage CAT conversion.

Table I. Activation of the different HPV URR CAT plasmids by the viral E2 proteins

	pJ4Ω	16E2	18E2B	BPV1E2
16URR::CAT	1.0	4.6 ± 2.2	3.0 ± 1.7	0.17 ± 0.04
18URR::CAT	1.0	3.4 ± 1.0	3.8 ± 0.6	ND
16URR:TK:CAT	1.0	3.6 ± 1.5	2.5 ± 0.6	3.7 ± 1.4

Numbers refer to fold activation and correspond to the mean of at least four independent experiments performed in normal primary human keratinocytes and AC89/E2 cells using 5 μg of the different pJ4ΩE2 constructs. ± indicates the standard error.
ND, not determined.

of activation. These results are in agreement with a previous report which showed that BPV-1 E2 strongly activated the HPV-18 URR in enhancer configuration but repressed transcription when the HPV-18 URR was placed in promoter configuration (Thierry and Yaniv, 1987). These data argue for a difference between the modes of action of the BPV and HPV E2 proteins on the HPV URR.

The results from the above series of experiments together with the standard deviations are summarized in Table I.

Overexpression of HPV-16 E2 results in non-specific transcriptional repression

The above analysis demonstrated that the full length HPV-16 E2 protein activated viral gene expression in cervical keratinocytes. We were then interested in determining the effects of high level E2 expression since this may also account for some of the reported differential activities. First, the HPV-16 E2 gene was transferred from pJ4Ω to pCMV. This plasmid contains the human CMV immediate early promoter which should be considerably more active in human cells than the murine LTR promoter in plasmid pJ4Ω. In order to verify this, pCMV-E2 and pJ4Ω16E2 plasmids were transfected into HT1080 cells. After 48 h the RNA was extracted and subjected to Northern blot analysis for E2 mRNA. The results obtained showed much

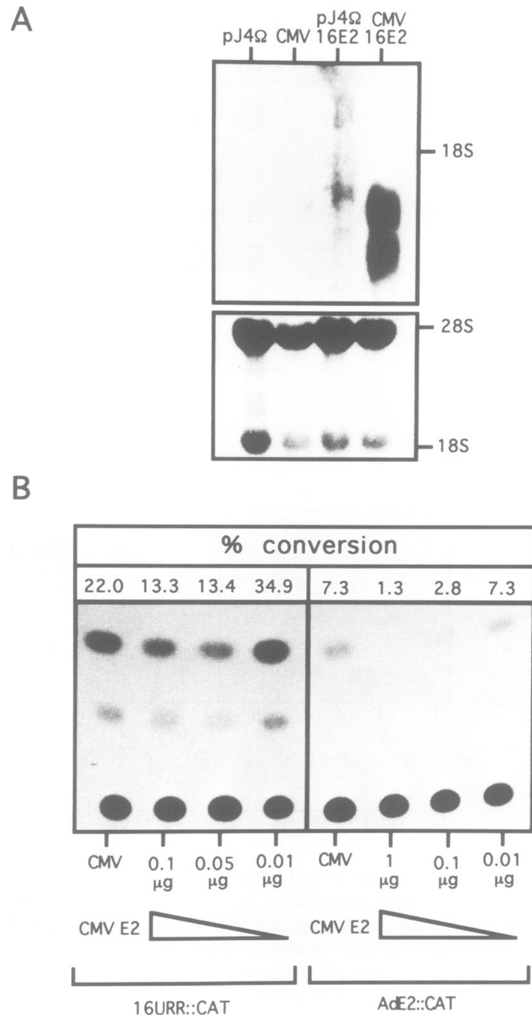

Fig. 6. (**A**) Northern blot analysis of E2 expression. HT1080 cells were transfected with the indicated plasmids. After 48 h the RNA was extracted and level of E2 mRNA expression determined by Northern blot analysis. The lower panel shows RNA loading on the same blot, which was detected using a random cDNA probe obtained by reverse transcription of total RNA. 18S and 28S RNAs are indicated. (**B**) Effect of high levels of HPV-16 E2 expression on 16URR::CAT and AdE2:CAT transcription. 7 μg of 16URR::CAT or 2 μg of AdE2::CAT plasmids were transfected into normal cervical keratinocytes at passage 2 together with either pCMV or the indicated amounts of pCMV-E2. Cells were harvested after 48 h and CAT activity measured. Numbers show percentage CAT conversion.

higher levels of E2 mRNA in the pCMV-E2 transfected cells than in the pJ4Ω16E2 transfected cells (Figure 6A).

We then proceeded to investigate the activity of the pCMV-E2 expression plasmid upon HPV-16 promoter activity. Normal human keratinocytes were transfected with pCMV-E2 together with 16URR::CAT and CAT activity was monitored after 48 h. The results obtained are shown in Figure 6B. Very low input levels of pCMV-E2 produced a dose dependent repression of HPV-16 promoter activity. These results indicated that high HPV-16 E2 expression will result in transcriptional repression of the HPV-16 promoter. In order to investigate the specificity of this activity we then assayed the effects upon the adenovirus E2 promoter which does not contain any E2 recognition sequences. The results of this analysis are also shown in Figure 6B and demonstrate sequence

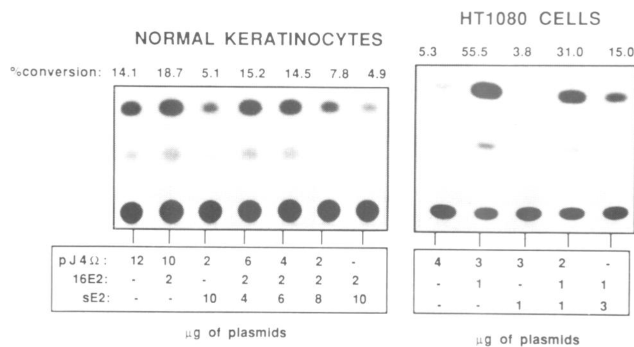

Fig. 7. Modulation of the transcriptional activation of pJ4Ω16E2 by pJ4Ω16sE2. The indicated plasmids, pJ4Ω, pJ4Ω16E2 and pJ4Ω16sE2 were transfected into normal keratinocytes (passage 2) and HT1080 cells in the quantities shown together with the HPV-16 URR:TK:CAT plasmid. After 48 h cells were harvested and CAT activity measured. Numbers show percentage CAT conversion.

independent transcriptional repression, reminiscent of squelching (Ptashne, 1988).

Differential splicing of the HPV E2 gene regulates viral gene expression

Recently a differentially spliced form of E2 was isolated from W12 cells (Doorbar *et al.*, 1990). This protein derives a few amino acids from the E1 region and the remainder consists of the C-terminal half of the E2 protein encoding the DNA binding and dimerization domains. This is similar to the E8^E2 repressor protein identified in BPV (Lambert *et al.*, 1987; Choe *et al.*, 1989; Vaillancourt *et al.*, 1990). Obviously we were interested in the transcriptional activity of this short form of E2, both by itself and in conjunction with the full length form of the E2 protein, since the C-terminal region of E2 had been previously shown to be capable of repressing viral promoter activity (Cripe *et al.*, 1987). Hence, the short E2 was cloned into pJ4Ω to produce pJ4Ω16sE2 (Figure 1). This was then assayed for any activity on the HPV-16 URR:TK:CAT plasmid in human cervical keratinocytes and HT1080 cells. In addition, a series of co-transfections were done with the full length form of the E2 protein pJ4Ω16E2 and pJ4Ω16sE2 in order to ascertain whether the two forms of E2 could alter their respective activities. Typical results are shown in Figure 7. It is clear from these experiments that at high input pJ4Ω16sE2 produced almost a 3-fold repression of HPV-16 promoter/enhancer activity in normal keratinocytes suggesting that the spliced form of E2 may have the ability to repress transcription by itself. More interesting however, is the effect of co-transfecting pJ4Ω16sE2 and pJ4Ω16E2 with the HPV-16 URR:TK:CAT reporter plasmid. In this case, increasing the amount of pJ4Ω16sE2 while retaining a constant input of pJ4Ω16E2 resulted in a progressive reduction in the transcriptional activation induced by pJ4Ω16E2. In normal keratinocytes, a 4:1 excess of pJ4Ω16sE2 over pJ4Ω16E2 resulted in transcriptional repression. A similar pattern of events is seen in HT1080 cells, although actual repression was not obtained under these conditions. These results define two distinct transcriptional activities for the HPV-16 E2 protein and indicate that the respective levels of the two forms of E2 are critical in controlling viral gene expression.

The dynamics of E2 trans-repression

Having characterized the transcriptional activity of the two different forms of E2 protein we proceeded to investigate potential mechanisms of action. One possibility was that the two forms of E2 were modulating each other's respective DNA binding activity, possibly through dimerization. To investigate this a series of gel retardation assays was performed on an oligonucleotide derived from the HPV-16 URR containing the two E2 recognition sequences placed immediately upstream of the p97 promoter. The two clones of E2 were transferred into SP64 and *in vitro* transcribed and translated before mixing with the labelled oligonucleotide. The results showed that both forms of E2 specifically retarded the labelled oligonucleotide (Figure 8A). The full length E2 protein–oligonucleotide complex migrated as a single discrete band. The upper unlabelled band on the gel, detected when using water primed lysate in the absence of E2 protein (data not shown), was not present upon addition of any antiserum (Figure 8B), and is therefore unrelated to E2. In contrast the short E2–oligonucleotide complex migrated faster and was composed of at least three different complexes. These results suggested that only one configuration of full length E2 was binding to the oligonucleotide, possibly as a tetramer, whereas the short form bound in a number of different configurations. We proceeded to investigate the ability of the different forms of E2 to produce heteromeric complexes since both forms retain the putative DNA binding and dimerization domains (Androphy *et al.*, 1987; McBride *et al.*, 1988, 1989; Moskaluk and Bastia, 1988). The two forms of E2 were *in vitro* translated in the same reaction mixture and then used in the gel retardation assay. The results obtained are shown in Figure 8B. As described above (Figure 8A), the full length and short forms of E2 migrated as distinct protein–oligonucleotide complexes. However, upon co-translating the two E2 proteins there was a marked shift in some of the retarded oligonucleotide, resulting in a complex which migrated with relative mobility between that of the full length and the short E2 complexes. This observation suggested that heterodimer formation had occurred. The identity of these complexes was confirmed by the supershift analysis using anti-E2 antibodies (kindly provided by Lutz Gissmann), the results of which are also shown in Figure 8B. These studies defined at least six different forms of E2–oligonucleotide complex and indicated the potential existence of a complex series of interactions between different E2 proteins and their DNA recognition sites during the regulation of viral gene expression.

As well as the formation of heteromeric complexes, regulation of viral gene expression could be mediated through competition between the two forms of E2 for the recognition sequence. Results of investigations are shown in Figure 8C where increasing amounts of short E2 were added to a constant input of full length E2 in the gel retardation analysis. These results showed that high levels of short E2 competed with the full length E2 protein for DNA binding. An additional point of interest from this result was the lack of heteromeric molecules. This suggests that the translated E2 proteins form very stable homomers with respect to the duration of the experiments; to obtain heteromeric molecules the proteins needed to be translated simultaneously.

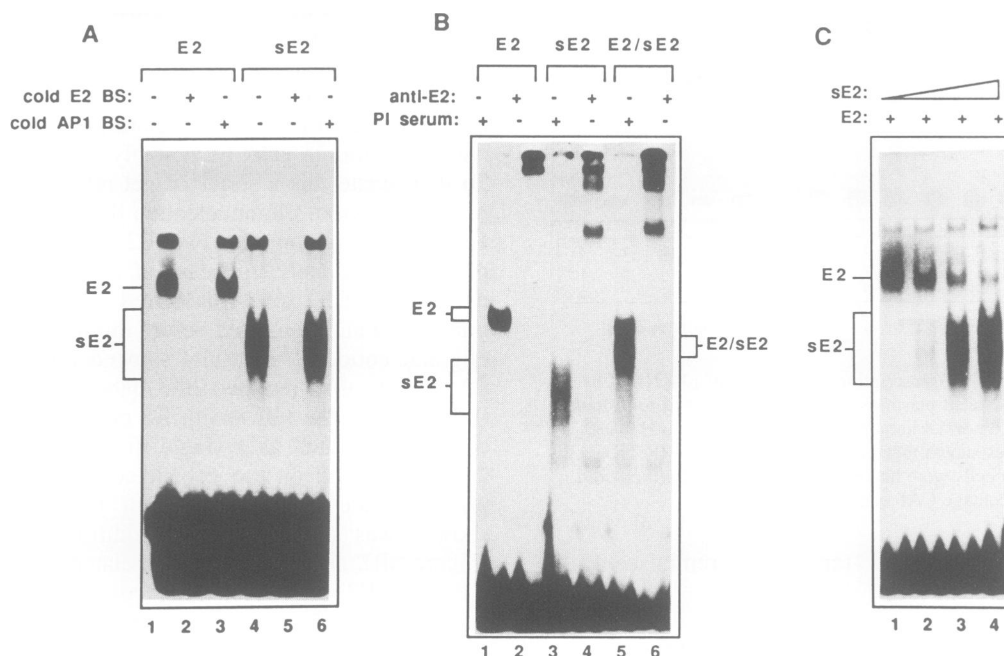

Fig. 8. Gel retardation analysis of a labelled E2 recognition site oligonucleotide with *in vitro* translated E2 proteins. (**A**) Full length HPV-16 E2 (E2) and short HPV-16 E2 (sE2) were *in vitro* transcribed and translated and incubated with labelled E2 recognition site oligonucleotide (E2 BS). Complexes were separated on non-denaturing PAGE. Shown are presence (+) or absence (−) of cold competing E2 BS oligonucleotide (cold E2 BS) or cold competing AP1 binding site oligonucleotide (cold AP1 BS). Retarded complexes corresponding to full length E2 (E2) and short E2 (sE2) are also indicated. (**B**) Gel retardation and supershift of E2 homomers and heteromers. HPV-16 full length E2 and short E2 were translated individually (tracks 1–4) or together (tracks 5 and 6) and incubated with labelled E2 BS oligonucleotide in the presence of pre-immune (PI) or anti-E2 serum as indicated. Full length E2 (E2) and short E2 (sE2) homomers and heteromers (E2/sE2) are indicated. Supershift can be seen in tracks 2, 4 and 6. (**C**) As for (A) except that a constant input (+) of full length E2 was maintained while increasing short E2 (sE2) (shown by triangle).

Finally we were interested in investigating the respective DNA binding affinities of the different forms of E2 molecule. To do this the E2 proteins were translated either individually or together and the retarded complex competed with increasing amounts of cold oligonucleotide. The results obtained are shown in Figure 9. We show that both the full length (Figure 9A), short (Figure 9B) and heteromeric forms (Figure 9C) of E2 have similar affinity for the recognition sequence.

Discussion

In the present study, we show unequivocally that the E2 proteins from HPV-16 and HPV-18 function as potent transcriptional activators of the viral promoter in normal keratinocytes. In addition, we demonstrate that a spliced form of E2, encoding the putative E2 repressor, represses the activity of the HPV-16 promoter/enhancer. Most significantly, co-transfection of the repressor form of E2 with the full length activator form results in a significant decrease in the ability of E2 to activate transcription. Gel retardation assays have defined a series of protein complexes between the different forms of E2 and suggest an elaborate mechanism for the control of viral transcription *in vivo* where the respective levels of the two E2 proteins are critical factors in the regulation of viral promoter activity.

The papillomavirus E2 proteins are major regulators of viral gene expression and represent an interesting group of transcription factors, displaying novel features in the mechanism of interaction with the DNA and in alternative splicing which regulates their activity. In BPV-1 the E2 protein is present in at least three different forms and exhibits transcriptional activation as well as repression depending on the form of E2 produced (Lambert *et al.*, 1987; Choe *et al.*, 1989; Vaillancourt *et al.*, 1990). The E2 binding motif is conserved throughout papillomaviruses and this has led to much speculation that the different E2 proteins would have a very similar function (Androphy *et al.*, 1987). In fact, studies that showed BPV-1 E2 capable of repressing HPV promoter activity tended to support this hypothesis (Thierry and Yaniv, 1987). However, little attention has been given to the activity of the HPV-16 E2 protein on its homologous promoter in the natural target cells. Here, in two different cervical keratinocyte systems, we have been able to show that the full length HPV-16 E2 protein strongly activates viral transcription. In comparison, under identical conditions BPV-1 E2 repressed HPV transcription. At present we cannot exclude the possibility that the BPV-1 E2 protein is more stable than the HPV-16 E2 protein and may give effects related to overexpression. However, using two additional reporter systems, TKM:32 and 16URR: TK:CAT, BPV-1 E2 gave levels of activation only slightly higher than that produced by HPV-16 E2. These results indicate that the expression levels of the two proteins are similar and, most importantly, demonstrate that BPV-1 E2 cannot be used as a model for HPV-16 E2 function on the HPV-16 promoter.

At this stage we were concerned by studies indicating that the full length HPV-18 E2 protein functioned as a repressor of the HPV promoter (Bernard *et al.*, 1989).

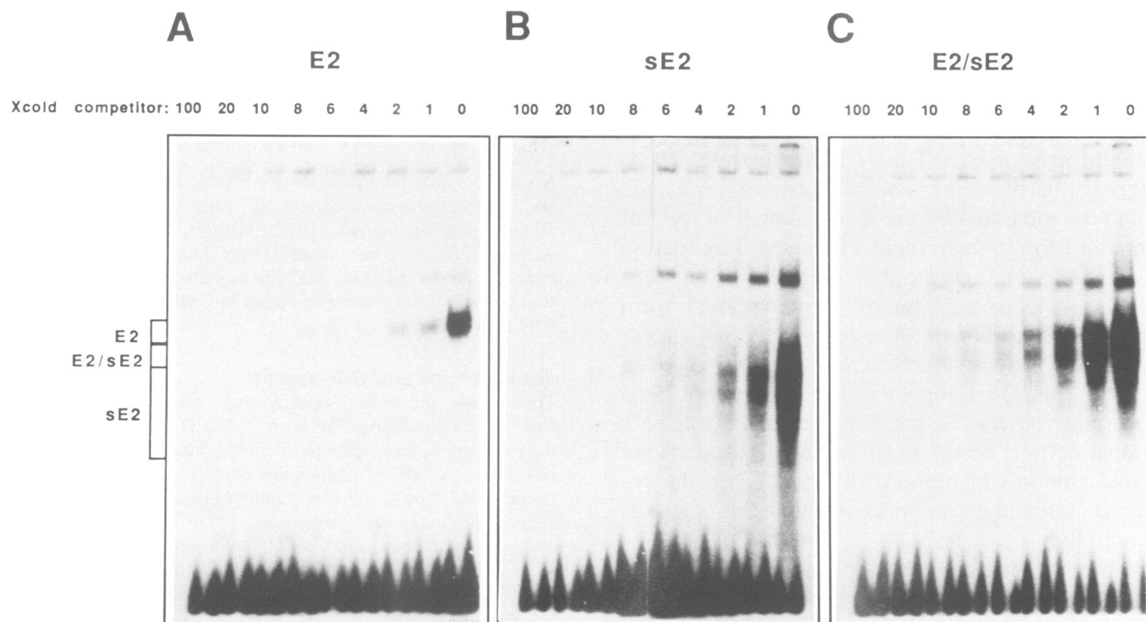

Fig. 9. Affinity of the different forms of HPV-16 E2 for the E2 recognition site. (**A**) Full length HPV-16 E2 (E2) was *in vitro* transcribed and translated. Constant input of the protein was incubated with labelled E2 BS oligonucleotide and decreasing amounts of cold E2 BS oligonucleotide (cold competitor). The numbers indicate the fold excess of cold competitor over the labelled E2 BS oligonucleotide (100×, 20×, 10×...etc., 0 meaning no cold competitor). Complexes were separated on non-denaturating PAGE. (**B**) Same as (A) except that a constant input of short HPV16E2 (sE2) was used. (**C**) Same as (A) except that constant input of the mixture containing the full length and the short HPV-16 E2 *in vitro* transcribed and translated together were used. Retarded complexes corresponding to full length E2 (E2) and short E2 (sE2) homomers and heteromers E2/sE2 are indicated.

To clarify the situation we constructed two different HPV-18 E2 expression plasmids; one corresponding to the plasmid previously described as a repressor and termed pJ4Ω18E2A in this study, and the second, containing a much shorter 5′ untranslated region, termed pJ4Ω18E2B. In our hands, pJ4Ω18E2A had minimal effect on the HPV-18 promoter, but in contrast, pJ4Ω18E2B always produced significant activation. Since the constructs encode the same E2 protein these differences are probably related to levels of expression. This conclusion is supported by the results using TKM:32 which demonstrate good activation by pJ4Ω18E2B but minimal activation by pJ4Ω18E2A. The results suggest that if the appropriate E2 expression system is used, then the full length protein behaves as a transcriptional activator similar to the HPV-16 E2 protein.

There are now several reports highlighting the importance of the E1 and E2 proteins in viral replication (Ustav and Stenlund, 1991; Yang *et al.*, 1991; Chiang *et al.*, 1992a,b). In many late stage cancers the HPV DNA is integrated into the host genome (Schwarz *et al.*, 1985; Baker *et al.*, 1987). This integration is often accompanied by deletions in viral sequences encoding E1 and E2. Thus, it is conceivable that in immortalization assays in which some viral DNA replication is possible, an increase in immortalization frequency would be expected upon mutation of either the E1 or E2 ORFs (Romanczuk and Howley, 1992). This would result in a virus which was more likely to become stably integrated into the host genome. However, *in vivo* viral integration is more likely at late stages of tumour progression in cells which cannot support the viral replication process (Matsukura *et al.*, 1989; Dürst *et al.*, 1992; Galehouse *et al.*, 1992). To attain this stage however, adequate levels of E6 and E7 proteins must be present in order to initiate the process of immortalization. We would suggest from our studies presented here and elsewhere (Lees *et al.*, 1990; Storey *et al.*, 1992) that functional E2 protein is an important factor in these early events. Only as the lesions progress to more advanced stages of dysplasia do we see additional cellular events taking place which render E2 activation unnecessary.

The pattern of HPV gene expression is complex, where the virus makes full use of alternative splicing to increase its coding potential. With HPV-16 this has been amply demonstrated by the isolation of a variety of different cDNAs from a keratinocyte line (W12) which was derived from a low grade cervical lesion and contains episomal HPV-16 DNA (Stanley *et al.*, 1989; Doorbar *et al.*, 1990). Of particular interest was the isolation of a cDNA encoding the C-terminal DNA binding domain of E2 but lacking the putative *trans*-activation domain. This cDNA is very similar to the BPV-1 repressor E2 cDNA E8^E2 described previously (Lambert *et al.*, 1987; Choe *et al.*, 1989; Vaillancourt *et al.*, 1990) and to several cDNAs isolated from other HPV-16 immortalized keratinocytes (Nasseri *et al.*, 1991; Rohlfs *et al.*, 1991; Sherman and Alloul, 1992). The studies presented here showed that this HPV-16 short E2 was capable of repressing the viral promoter/enhancer, indicating intrinsic repressor activity. Perhaps more importantly, we showed that the short E2 could interfere with the transcriptional activation of the full length HPV-16 E2 protein. These results define two separate activities for HPV-16 E2 and suggest that *in vivo* the respective amounts of the two forms of E2 are critical for controlling viral gene expression.

Finally we were interested in how the different activities of E2 might be brought about. We showed that both the

full length and short E2 proteins were capable of binding an oligonucleotide containing two E2 recognition sites. A series of competition experiments demonstrated that, although the different forms of E2 had similar DNA binding affinities, excess short E2 protein could compete the full length form of E2 off the oligonucleotide. These results suggest that the repression of transcription by the short E2 may be mediated by the displacement of the full length activator form of the protein. However, the situation is not as simple as it may at first seem, since *in vitro* translated E2 proteins may rapidly dimerize and form homomeric complexes which, once formed, do not appear to reassociate and form heteromeric complexes. However, when *in vitro* translated together, the E2 proteins can readily form heteromers. At present we cannot ascribe a transcriptional activity to the heteromeric E2 protein, but from the low amounts of homomeric short E2 in the co-translations it is tempting to speculate that this also has repressor activity.

Based on these studies it is now important to differentiate between the different forms of E2 produced during the course of a normal viral infection and at different stages of transformation. In particular, the relative levels of the proteins will have a crucial bearing on the levels of expression of the viral oncoproteins, E6 and E7. Studies are also in progress to define the transcriptional activities of the heteromeric E2 proteins.

In conclusion, the present study has characterized two different regulatory activities for the HPV-16 E2 protein in human keratinocytes. These studies redefine the role of the E2 gene products in viral replication and cell transformation and demonstrate a more complex series of interactions than was hitherto considered.

Materials and methods

Plasmids

The HPV E2 expression plasmids used in this study were constructed as follows and are also shown in Figure 1. Full length E2 was cloned into pJ4Ω to give pJ4Ω16E2 as described previously (Lees *et al.*, 1990) and consists of nucleotides 2755–3853 from the HPV-16 genome. The short E2 expression plasmid pJ4Ω16sE2 contains HPV-16 E2 sequences from coordinates 3357 to 4213 of the viral genome. This was derived from a cDNA (Doorbar *et al.*, 1990) obtained from the W12 cell line (Stanley *et al.*, 1989). This derives 21 bp from the E1 region at its N-terminus and is only capable of producing the C-terminal half of the E2 protein (Doorbar *et al.*, 1990). pCMV-E2 consists of the full length E2 from HPV-16 cloned from pJ4Ω into the *Bam*HI site of pCMV. Two HPV-18 E2 expression plasmids were used in this study. pJ4Ω18E2A was cloned by excising the *Nsi*I fragment from HPV-18, blunt ending and ligating into the *Hin*dIII site of pJ4Ω. This construct is analogous to the HPV-18 E2 expression plasmid described previously (Bernard *et al.*, 1989). pJ4Ω18E2B was constructed by cutting pJ4Ω18E2A with *Tth*III1, blunt ending and excising with *Bam*HI. This was then recloned into *Sma*I/*Bgl*II restricted pJ4Ω. This construct has 75 bp fewer than pJ4Ω18E2A prior to the first ATG of the E2 gene. The BPV-1 E2 gene was obtained from the BPV-1 genome by PCR amplification using primer CCCGGATCCGAAGAGGATGGAGACAGC located just prior to the E2 start position 2601 and primer CCCGAATTCTCAGAAGTCC-AAGCTGGC located just at the end of the E2 codon position 3839, and was cloned into the *Bam*HI/*Eco*RI restriction site of pJ4Ω plasmid to give the construct pJ4ΩBPV1E2.

The CAT reporter plasmids used in these studies consist of the HPV-16 URR from nucleotides 7403 to 114 cloned into the *Hin*dIII/*Bam*HI sites of pBLCAT 3 (Luckow and Schutz, 1987) and termed 16URR::CAT. The same 16URR fragment cloned into the *Hin*dIII/*Bam*HI sites of pBLCAT 2 where the CAT gene is under the control of the TK promoter is termed 16URR:TK:CAT. The HPV-18 URR from nucleotides 6930 to 120 cloned into the *Bam*HI site of pBLCAT3 is termed 18URR::CAT. The latter construct is analogous to the previously described reporter plasmid for the HPV-18 URR (Bernard *et al.*, 1989). The TKM:32 construct containing 6× E2 binding sites and a TK promoter uptream of the CAT gene was a kind gift of Moshe Yaniv and Françoise Thierry (Thierry *et al.*, 1990).

Cells

Normal cervical keratinocytes were obtained from hysterectomies for non-HPV related disease, and the cells were cultured as described previously (Storey *et al.*, 1992). HPV-16 immortalized keratinocytes AC89/E2 cells have been described previously (Storey *et al.*, 1992). The human sarcoma cell line, HT1080 has also been described previously (Laug *et al.*, 1983) and was cultured in DMEM supplemented with 10% fetal calf serum.

Transfections and CAT assays

Transfections of normal keratinocytes, and AC89/E2 cells were performed with polybrene (Farr *et al.*, 1987). HT1080 cells were transfected by standard calcium phosphate precipitation as described previously (Wigler *et al.*, 1979). Cells were transfected with 7 μg CAT reporter plasmid and 5 μg of E2 expression plasmid unless otherwise indicated. Where titrations were performed, DNA input was standardized by addition of increasing amounts of vector only DNA. After 48 h the cells were harvested in 150 μl CAT buffer (40 mM Tris–HCl pH 7.5, 150 mM NaCl; 1 mM EDTA) and subjected to three cycles of freeze–thawing, followed by incubation at 65°C for 10 min. Samples were clarified by centrifugation at 14 000 r.p.m. for 2 min in an Eppendorf centrifuge and protein concentration of the supernatant was measured by the Bio-Rad protein assay. CAT assays were routinely performed with 5–10 μg of protein incubated with 2.5 μl acetyl-CoA (33.3 mg/ml) and 1.5 μl [^{14}C]chloramphenicol (50 mCi/mmol; Amersham) in a final volume of 100 μl at 37°C for 1 h. Following extraction with ethyl acetate, samples were analysed by thin layer chromatography and visualized by autoradiography. Quantitation was performed by excising the products and liquid scintillation counting.

Reverse transcription and PCR amplification

One μg of total RNA harvested 48 h after polybrene transfection of AC89/E2 cells with 16URR::CAT plus either pJ4Ω or pJ4Ω16E2 was reverse transcribed using the primer GGATATATCAACGGTGGTA at position 79–60 in the CAT gene. Three μl of the reaction were amplified by PCR using the above primer and one of the three primers located within the HPV-16 URR. These are 18mers starting at positions 62, TAGTATAAAAGCAGACAT; 80, TTTATGCACCAAAAGAGA and 97, AACTGCAATGTTTCAGGA. PCR products were run on a 12% polyacrylamide gel.

In vitro translations and gel retardation assays

HPV-16 E2 proteins were expressed *in vitro* from SP64 using the TNT coupled (SP6) reticulocyte lysate system (Promega). Oligonucleotides corresponding to two E2 recognition sites, 5′-GCTTCAACCGAAAT-CGGTTGAACCGAAACCGGTTGCATG-3′, were labelled in the presence of [γ-^{32}P]ATP (Amersham, UK) and polynucleotide kinase. Labelled oligonucleotides were incubated at room temperature with *in vitro* translated protein for 20 min. For supershift analysis the polyclonal anti-E2 antiboy raised against the C-terminal domain of the protein (kindly provided by Lutz Gissmann) was added to the E2–oligonucleotide complex after the former 20 min binding reaction and the mixture incubated for a further 30 min at 4°C. The complexes were resolved on non-denaturing 6% acrylamide (acrylamide:bis, 55:1) gels in TBE. The gels were dried and processed by autoradiography.

Northern blot analysis

Northern blot analysis was carried out as described previously (Storey *et al.*, 1988). Equal loading of RNA was confirmed using a random cDNA probe obtained after reverse transcription of total RNA.

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