An element binding a C/EBP-related transcription factor contributes to negative regulation of the bovine papillomavirus type 4 long control region

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Deletion of the NR2 element of the long control region (LCR) of bovine papillomavirus type 4 (BPV-4) was observed previously to lead to a fivefold increase in enhancer activity of a subfragment of the LCR. Further characterization of this element indicates that mutations in NR2 lead to increased enhancer activity in both mouse CT3 fibroblasts and in a transformed bovine epithelial cell line derived from an alimentary canal papilloma/in situ carcinoma, but not in primary bovine keratinocytes. Since similar oligonucleotide–nuclear factor complexes were obtained in electrophoretic mobility shift assays (EMSA) for all three cell types, the observed difference in negative activity may result from variation in the NR2-binding factor itself between primary and established/transformed cell lines, or from the involvement of other factors that vary between the lines. Characterization of the NR2-binding factor by heat stability and antibody supershifts in EMSA indicate that the factor is related to the CCAAT/enhancer-binding protein (C/EBP) family, and that one component of the complexes may be C/EBPβ.

Introduction

Bovine papillomavirus type 4 (BPV-4) infection results in the formation of alimentary canal papillomas that can progress to carcinoma in cattle feeding on bracken fern, a source of both carcinogens and immunosuppressants (Campo & Jarrett, 1986). The viral genome encodes proteins that, in cooperation with an activated ras gene, can transform primary bovine cells in culture (Jaggar et al., 1990; Pennie et al., 1993). The frequency of in vitro transformation is increased by a high level of expression of the viral E8/E7 open reading frames (ORFs), suggesting that transcriptional control of the viral genome by viral and/or host factors may be an important determinant in progression to carcinoma.

Evidence is beginning to accumulate indicating that negative regulation of viral transcription by host factors is important in the normal viral life cycle and also plays a role in progression to carcinoma. Thus the transcriptional enhancer of human papillomavirus type 8 (HPV-8) is active in skin fibroblasts of patients with epidermodysplasia verruciformis (EV) and in several human carcinoma cell lines, but inactive in normal human keratinocytes or fibroblasts, suggesting that the cells of both EV patients and carcinomas had either gained a factor required for HPV-8 enhancer activity, or that these cells lacked a negative factor which suppressed the viral enhancer (Fuchs & Pfister, 1990). Potential targets for such factors are the positive and negative cis-acting sequences shown to be present in the long control region (LCR) of HPV-8 (Reh & Pfister, 1990). Overexpression of the ubiquitous cellular transcription factor Oct-1 has been found to repress HPV-18 expression, although the mechanism of this repression is unclear as an Oct-1–DNA interaction was not involved (Hoppe-Seyler et al., 1991). Epidermal growth factor (EGF) has been shown to elicit down-regulation of HPV-16 transcription via an EGF-responsive element in the viral LCR (Yasumoto et al., 1991). Protein phosphorylation has been shown to be important in regulation of viral gene expression as inhibition of protein phosphatase 2A (PP2A) either by simian virus 40 (SV40) small t antigen or by increased expression of the 55K regulatory subunit of PP2A results in trans-activation of the HPV-16 LCR (Smits et al., 1988, 1992a, b). Evidence is also beginning to accumulate for the existence of cellular ‘silencing’ functions that repress papillomavirus transcription. Thus, a cis-acting silencer element has been identified in the LCR of HPV-6 (Wu & Mounts, 1988) and the transcription factor YY1 interacts with negative elements in the LCRs of HPV-16 and -18 (Bauknecht et al., 1992; May et al., 1994). Rosl et al. (1991) demonstrated that transcription from the HPV-18 LCR in the SiHa cervical carcinoma line was abolished following cell fusion with a non-tumorigenic keratinocyte line; the latter was postulated to provide trans-acting negative regulatory factors.
absent from the carcinoma cells. Thus several cellular factors have to date been implicated in negative regulation of papillomavirus transcription.

Both positive [CE1, CE2, CE3 (CE, control element)] and negative [NR1, NR2, NR3 (NR, negative regulator)] transcriptional control elements occur in the LCR of BPV-4 (Fig. 1; Jackson & Campo, 1991); these elements respond to cellular factors and therefore represent potential targets for cellular control of viral gene expression. The results described here indicate that a CCAAT/enhancer-binding protein (C/EBP)-related nuclear factor may be involved in the negative regulation of BPV-4 LCR activity through the NR2 element.

**Methods**

**Cell culture.** CT3 cells (a line of NIH3T3 cells obtained from Geoffrey M. Cooper) and 88529B cells (transformed bovine epithelial cells derived from an alimentary papilloma/in situ carcinoma) were cultured in SLM (Gibco BRL) supplemented with 10% fetal calf serum (FCS; Gibco BRL). PalK cells (primary fetal bovine palate keratinocytes) were derived from fetal palate biopsies using the method described by Cuthill et al. (1993) for human cervical keratinocytes. These cells were cultured on irradiated 3T3 feeder layers in SLM supplemented with 10% FCS, 0.1 nm-cholera enterotoxin, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 180 μg-adenine and 10 ng/ml EGF (all from Sigma).

**Transfection.** Transfections of CT3 cells were carried out by calcium phosphate precipitation exactly as described by Gorman (1985) using 10 μg of test plasmid and 5 μg of control plasmid pHSV-β-gal, containing the Escherichia coli β-galactosidase ORF under the transcriptional control of the herpes simplex virus (HSV) IE4 promoter. The precipitates were left on the cells for 18 hr before washing; the cells were harvested 30 hr after transfection. 88529B cells and PalK cells (plated without feeder cells) were transfected by polybrene-DMSO treatment essentially as described by Jiang et al. (1991), using 10 μg of test plasmid and 5 μg of control plasmid pCH110, which carries β-galactosidase downstream of the SV40 early promoter. Cells were plated at 2 x 10^4 (88529B) or 10^5 (PalK) per 25 cm² flask and centrifuged in an Eppendorf centrifuge for 15 min at 4 °C. The pelleted nuclei were resuspended in 25 μl buffer B (20 mM-Tris pH 7.5, 1.5 mM-MgCl₂, 0.2 mM-EDTA, 20 mM-KCl, 25% glycerol and protease inhibitors as described for buffer A) and then 100 μl buffer C (buffer B containing 600 mM-KCl) was added dropwise. The preparations were incubated on ice for 30 min, centrifuged in an Eppendorf centrifuge for 15 min at 4 °C and the supernatant stored at −70 °C. The protein concentrations of the extracts ranged from 1.5 to 40 μg/μl.

**Electrophoretic mobility shift assays (EMSA).** Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer, purified on denaturing polyacrylamide gels, annealed and labelled with ³²P using Klenow polymerase or T4 polynucleotide kinase using standard protocols (Sambrook et al., 1989). Oligonucleotide sequences were as follows: NR2, 5’ TCGAGTTTGTGCAACCAGAGCTCTTCAAACTTGCAGAC 3’ and C’ ACAAAACGGTTGCGCGGAAGCTTTGACCTGTGACACCTCT 5’. C/EBP, 5’ GATCGAAGGGCATCCATGCTGAC 3’. AATCAGGGCTACCCATTTTGGATGAGCT 5’. Mutant NR2 oligonucleotides contained the mutations shown in Fig. 2 incorporated into the above sequences. The C/EBP-binding site is as described by Landschulz et al. (1988) and the EFII oligonucleotide represents the sequences (nt) 3229 to 192 of the Rous sarcoma virus (RSV) LTR as described by Sears & Sealy (1992).

DNA binding reactions were performed using 5 to 10 μl nuclear extract and 1 μg poly(dIC) in 12 mM-HEPES pH 7.9, 100 μg/ml BSA, 1 mM-DTT, 50 mM-NaCl, 5 mM-EDTA, 12% glycerol. After a 10 min preincubation on ice 0.01 pmol labelled oligonucleotide and 1 pmol of unlabelled competitor oligonucleotides where appropriate were added and incubation continued at room temperature for 15 min. Antibody supershifts were performed by the addition of 2 μl of rabbit anti-C/EBP serum (Santa Cruz Biotechnology, Inc.) to the reaction and incubation on ice for a further 75 min. Samples were electrophoresed on 6% polyacrylamide gels at 9 V/cm in 300 mM-glycine, 50 mM-Tris, 50 μM-Tris, 1 mM-EDTA, pH 8.7. For heat stability tests the nuclear extracts were incubated at 50 °C for 5 min or 90 °C for 15 min and then cleared by centrifugation for 10 min at 12000 r.p.m. in an Eppendorf centrifuge at 4 °C.

**Methylation interference.** The NR2 oligonucleotide was labelled with [³²P]ATP on one strand only and partially methylated at guanine residues with dimethyl sulphate as described by Maxam & Gilbert (1980). DNA-protein complexes and free probe from a 20-fold scaled up nuclear factor-binding reaction were separated by preparative acrylamide gel electrophoresis and transferred to NA45 paper (Schleicher & Schuell) by electroblotting. Complexes were visualized by autoradiography, excised and eluted by a 3 h incubation at 65 °C in 10 mM-Tris pH 7.9, 1 mM-EDTA, 1 mM-NaCl. Following ethanol precipitation the oligonucleotides were cleaved at methylated residues with piperidine as described by Maxam & Gilbert (1980). The products were analysed by electrophoresis on an 18% polyacrylamide gel containing 8 M-urea.

**Results**

A cellular factor binds specifically to the inverted repeat motifs of NR2

Three negative regulatory elements, NR1, NR2 and NR3, were identified in the LCR of BPV-4 by enhancer assays of deleted LCR derivatives (Fig. 1; Jackson &
Fig. 1. (a) Structure of the BPV-4 LCR. The locations of DNaseI footprints and positive (CE1, -2 and -3) and negative (NR1, -2 and -3) control elements identified by earlier analyses (Jackson & Campo, 1991) are indicated. Filled circles represent E2 consensus binding sites and the open circle represents a degenerate E2 site. C, cytokeratin octamer; N, NF1 homology; T, TATA box. (b) Sequence of NR2CE2 (HpaII to BglII); the BstNI site at nt 15 (CCTGG) used for subcloning CE2 is underlined. The inverted repeats of NR2 and the locations of DNaseI footprints are indicated; the sequences overlapping FP6 and FP7 which correspond to FP9 in BPV-4 CE1 and the BPV-1 5’ enhancer respectively are underlined.

Campo, 1991). A subclone of the LCR from nt 7259 to 310 was demonstrated to possess only one-fifth of the activity of a similar clone from which the 25 bp defining NR2 had been deleted. None of the DNaseI footprints identified in this study mapped to the NR2 element although DNaseI hypersites could be observed in this region at high protein concentrations. The NR2 element possesses sequence motifs (Fig. 2a) that might represent nuclear factor-binding sites, i.e., a 7 bp inverted repeat for which the two halves are separated by a GC box. The 5’ inverted repeat overlaps a second inverted repeat of 4 bp.

Nuclear factor binding to NR2 was analysed by EMSA. Nuclear extracts were prepared from mouse CT3 fibroblasts, PalK cells and tumorigenic bovine 88529B epithelial cells. An NR2 oligonucleotide probe was incubated with the nuclear extracts and found to form three major retarded complexes (I, II and III) in EMSA; the retarded complexes were shown to be specific by competition with excess unlabelled oligonucleotide (Fig. 3). Nuclear extracts from all three cell types gave identical results, indicating that the NR2-binding factor is present in both fibroblasts and keratinocytes. The ability to detect an NR2-binding factor by EMSA when no DNaseI footprint had been previously observed is probably a result of the greater sensitivity of EMSA relative to DNaseI footprinting. Several mutant forms of the NR2 oligonucleotide were tested in the gel-shift assay; the results are summarized in Fig. 2(a). NR2mt1, in which three substitutions were made in each half of the inverted repeat, was unable either to produce a specific retarded complex or to out compete the complexes formed by NR2 (Fig. 2a, Fig. 3). NR2mt2, in which 4 bp within the GC box were substituted with A-T base pairs while the inverted repeats remained intact, was able both to form specific retarded complexes of the same mobility as those formed by NR2, and to compete out the complexes formed by NR2 (Fig. 2a). Thus it appeared that the inverted repeat sequences rather than the GC box were important for nuclear factor binding. Both NR2mt4 and NR2mt7, in which only the 5’ repeat was substituted, and NR2mt8 with substitutions only in the 3’ repeat, were unable either to form specific retarded complexes or to compete out the complexes formed by NR2. However, NR2mt6 with the same set of substitutions in the 3’ repeat as are present in the 5’ repeat in NR2mt4, retained the ability to form specific retarded complexes and compete out the complexes formed by NR2, although the efficiency of this latter competition was variable and may be indicative of a somewhat weaker binding of nuclear factor(s) to NR2mt6 than to NR2. The results suggested that, although both repeats were involved, the 5’ repeat was more important than the 3’ repeat for nuclear factor binding. This was confirmed using methylation interference.

Identification by methylation interference of G residues important for nuclear factor binding to NR2

NR2 oligonucleotides were [32P]end-labelled on one strand only and partially methylated at guanine residues as described in Methods. Free and retarded oligonucleotide bands were cut from the acrylamide gel, cleaved at methylated guanines using piperidine and the products run on a sequencing gel. Identical methylation interference patterns were obtained for the three NR2 retarded complexes; the results for complex I are shown in Fig. 4. Three G residues [indicated by filled circles in Fig. 2(b) and filled stars in Fig. 4] are present within the 5’ repeat for which methylation severely impairs nuclear factor binding to the oligonucleotide. There are two additional guanine residues adjacent to the 5’ repeat
Fig. 2. (a) The NR2 sequence is shown with the 7 and 4 bp inverted repeats indicated by arrows. The sequences of the mutants are indicated. The results of EMSA for NR2 and its mutants are summarized; ++ + + + indicates wild-type levels of DNA-protein complex formation by mutant oligonucleotide and the ability to compete out the complexes formed by wild-type NR2; + + + + indicates complex formation but a reduced ability to compete out the complexes formed by wild-type NR2; -- indicates an inability both to form DNA-protein complexes and to compete out the complexes formed by wild-type NR2. (b) the G residues for which methylation interferes with NR2 factor binding are indicated on the NR2 sequence. Filled circles indicate a loss of binding following methylation and open circles indicate reduced binding (see Fig. 3). (c) Comparison of the inverted repeats of NR2 and EYI with the C/EBP consensus (Ryden et al., 1993). (d) Alignment of NR2 and EYI sequences indicating the inverted repeats for each sequence and a gapped direct repeat for EYI.

The C/EBP oligonucleotide formed retarded complexes of similar mobility to those formed by the NR2 oligonucleotide, and these complexes could be competed out by excess unlabelled NR2. These results indicated that the C/EBP and NR2 oligonucleotides were binding the same nuclear factor(s). However, in PalK extracts NR2 consistently competed relatively poorly with the C/EBP oligonucleotide for nuclear factor binding, which may suggest a qualitative difference in the NR2-binding factor in PalK cells. Another potential C/EBP-binding site (TGTTAAG) maps to FP7 within the CE2 enhancer adjacent to NR2 (Fig. 1), although the FP7 oligonucleotide was unable to compete out the NR2 complexes (Fig. 5a) and formed complexes of a different mobility to those formed by NR2 (data not shown).

The NR2-binding factor appears to be related to C/EBP

The NR2 element shows some resemblance to the consensus binding site for the C/EBP family (Fig. 2c), and, as shown in Fig. 5a, a C/EBP oligonucleotide was able to compete with the NR2 oligonucleotide in EMSA.
C/EBP binding to BPV-4 LCR

II, suggesting that complex III contains heat labile components.

The ability of anti-C/EBP antibodies to produce supershifts in EMSA was tested using polyclonal antisera specific for C/EBPα, C/EBPβ and C/EBPδ. Anti-C/EBPβ, but not anti-C/EBPα or anti-C/EBPδ, was able to shift the mobility of all three NR2 complexes formed with CT3 (Fig. 6b), 88529B and PalK (not shown) nuclear extracts, indicating that one component of these complexes is C/EBPβ or is at least more closely related to C/EBPβ than to C/EBPα or C/EBPδ.

The EFII element of the RSV long terminal repeat (LTR) which shows considerable homology to NR2 (Fig. 2c, d) binds both C/EBPα (Ryden & Beemon, 1989) and a C/EBP-like factor found in fibroblast and keratinocyte cell lines (Sears & Sealy, 1992). An oligonucleotide corresponding to the EFII element was therefore tested for binding and competition in PalK nuclear extracts (Fig. 5b). The EFII oligonucleotide produced a comparable profile of retarded complexes in PalK extracts to that obtained by Sears & Sealy (1992) using chick embryo extracts. EFII was able to compete with NR2 in EMSA, and similarly, NR2 could also compete for nuclear factor binding by EFII. However, whilst EFII was able to compete out all three NR2 complexes, NR2 competed very inefficiently for the low mobility EFII complexes, even at a 500-fold excess. Identical results were obtained for nuclear extracts from CT3 and 88529B cells.

Effect of the NR2 and NR2mt1 sequences on the enhancer activity of the BPV-4 CE2 element in CT3 and PalK cells

Deletion analysis of the BPV-4 LCR identified a positive element, CE2, the removal of which led to a considerable drop in LCR enhancer activity (Fig. 7; Jackson & Campo, 1991). Binding sites for two nuclear factors within the CE2 element (FP6 and FP7) were identified by DNaseI footprinting. FP6 mapped to a sequence showing a 10 out of 12 nt match to footprint FP9 in enhancer CE1 of the BPV-4 LCR (Jackson & Campo, 1991) and FP7 maps to a sequence showing homology to the BPV-1 5' enhancer (Spalholz et al., 1985). Since CE2 lies adjacent to the NR2 element, the effects of NR2 on the enhancer activity of CE2 were tested using the enhancer assay plasmid p41X (Jackson & Campo, 1991) which carries a CAT gene under the transcriptional control of the HSV...
The CE2 element was subcloned as a 67 bp *Bst*NI–*Bal*I fragment (nt 15 to 81; Fig. 1), and includes FP7, FP6 and part of FP5 that represents a weak homology to NF1. This fragment was inserted upstream of the TK promoter in p41X in single or multiple copies and the resultant plasmids were transfected into CT3 cells for which CAT expression was measured. Single copies of CE2 placed in either orientation upstream of the TK promoter had no significant effect on promoter activity (Fig. 7). However, when present as two or more copies in either orientation the individual CE2 elements synergized to increase TK promoter activity, thus acting as enhancers (Fig. 7). To test the effect of NR2 on CE2 activity, NR2 and CE2 were subcloned together as a 104 bp *Hpa*II–*Bal*I fragment, NR2CE2 (nt 7243 to 81). In addition, in vitro mutagenesis was used to introduce the base substitutions of oligonucleotide NR2mt1 that abolished nuclear factor binding in EMSA (Fig. 2, Fig. 3), into the NR2 element to give NR2mt1CE2. Both NR2CE2 and NR2mt1CE2 were inserted as single or multiple copies into p41X and the levels of CAT expression following transfection into CT3 cells were measured. When NR2CE2 or NR2mt1CE2 were present as one or two copies in either orientation very low enhancer activity was observed and there was little difference in CAT expression between the pairs of plasmids (not shown). However, with 4 × NR2CE2+ a CAT activity of 1.74-fold that of the vector control was obtained compared to a value of 3.92-fold that of the vector control with 4 × NR2mt1CE2+. Similarly, with 5 × NR2CE2− the CAT activity was equivalent to the vector control whereas with 5 × NR2mt1CE2− the CAT activity was 2.16-fold that of the vector control. Thus, for multimers of four and five copies, CAT expression from plasmids containing the mutant version of NR2 was significantly increased relative to that from plasmids containing the wild-type NR2. Since NR2mt1 has lost a nuclear factor binding function by EMSA it would appear that the function of this nuclear factor is to downregulate transcription.

The oligonucleotides NR2 and NR2mt1 (Fig. 2) were inserted upstream of the TK promoter in p41X to establish the effects of these sequences in the absence of enhancer elements. Constructs containing five tandem copies of either NR2 or NR2mt1 expressed similar low levels of CAT, slightly less than that obtained from the TK promoter alone (Fig. 7). Two conclusions could be drawn from these results: firstly, that multiple copies of NR2 had no significant effect on expression from the enhancerless TK promoter, and secondly, that NR2mt1 had not gained an enhancer function.

In PalK cells the CE2 element showed greater enhancer activity compared to that in CT3 cells when present in single or multiple copies (Fig. 7). However, no significant
Fig. 6. (a) Heat stability of NR2-binding factor in CT3, PalK and 88529B extracts. Nuclear extracts were heat-treated as shown before EMSA using an NR2 probe; unlabelled NR2 competitor was added at a 100-fold excess in the lanes indicated. Arrowheads show the positions of complexes I, II and III. Note: The untreated 88529B extract produced the normal profile of three specific complexes with the NR2 probe, but the autoradiograph is an overexposure for this lane to give an indication of the proportion of active protein remaining following heat treatment. (b) Antibody supershifts of NR2 complexes formed with CT3 nuclear extract. Incubations were performed with the following additions: Lane C, 100-fold unlabelled competitor oligonucleotide; lane 0, no addition; lane PI, preimmune rabbit serum; lane π, rabbit anti-C/EBPa; lane β, rabbit anti-C/EBPβ; lane δ, rabbit anti-C/EBPδ. S indicates the supershifted complexes.

Fig. 7. Enhancer activities in CT3 and PalK cells of CE2, NR2CE2, NR2mt1CE2, NR2 and NR2mt1 inserted as single or multiple copies upstream of the TK promoter and CAT-coding sequences in p41X (Fig. 8). In mouse CT3 fibroblasts the wild-type LCR in either orientation stimulated transcription from the TK promoter by approximately threefold. LCRmt7/6 and LCRmt7/8 gave an approximately four- to fivefold stimulation, irrespective of LCR orientation. Thus mutation of the NR2 element within the context of the intact LCR significantly increased enhancer activity. However, LCRmt4/8 showed a decreased enhancer activity with respect to the wild-type LCR.

Wild-type LCR enhancer activity was almost undetectable in the transformed bovine epithelial line 88529B, but the introduction of NR2 mutations 7/6 and 7/8 into the LCR led to a significant level of enhancer activity being observed (Fig. 8). As in CT3 cells, LCRmt4/8 had a similar activity to the wild-type LCR. In PalK cells all of the mutants had similar or lower enhancer activities than

Fig. 8. Enhancer activities in CT3 and 88529B cells of the wild-type LCR (at 6711 to 331) and derivatives in which the NR2 element has been mutated. Other details as in the legend to Fig. 7.

**Effect of NR2 mutants on LCR enhancer activity**

Mutations 4/8, 7/6 and 7/8 were introduced into the BPV-4 LCR cloned in both orientations upstream of the TK promoter and CAT-coding sequences in p41X (Fig. 8). In mouse CT3 fibroblasts the wild-type LCR in either orientation stimulated transcription from the TK promoter by approximately threefold. LCRmt7/6 and LCRmt7/8 gave an approximately four- to fivefold stimulation, irrespective of LCR orientation. Thus mutation of the NR2 element within the context of the intact LCR significantly increased enhancer activity. However, LCRmt4/8 showed a decreased enhancer activity with respect to the wild-type LCR.

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that of the wild-type LCR (not shown), again indicating a functional difference in the NR2 factor between cell types.

Discussion

Multimerization of NR2 together with CE2 at four or five copies indicated that NR2, but not the mutant derivative, was able to suppress the enhancer activity of CE2, but did not completely eliminate it (Fig. 7). However mutation of the NR2 element had no effect on CE2 enhancer activity in PalK keratinocytes. Thus, although comparable NR2–nuclear factor complexes were formed by nuclear extracts from CT3 and PalK cells, the functional activity of NR2 varied between the cell types. Similar results were obtained when NR2 increased enhancer activity with respect to the wild-type element in CT3 and 88529B cells but was inactive in PalK cells. Since NR2mt7/6 and NR2mt7/8 oligonucleotides had lost the ability to bind nuclear factors in vitro (Fig. 2) and showed no enhancer activity in vivo in any of the cell types when multimerized (data not shown) it was concluded that a negative factor functioned via the NR2 element in CT3 and 88529B cells but was inactive in PalK cells. The lower enhancer activity observed for LCRmt4/8 was unexpected and perhaps indicates that a positive factor may also act through the NR2 element. The complex structure of the NR2 region makes it possible that several different nuclear factors may bind this element and it is conceivable that a positive factor binding at NR2 requires to act with a second factor binding an adjacent site to act as an enhancer. The sequence A/GCACCCG occurs both within NR2 and in the late promoters of several skin-specific papillomaviruses (Stubenrauch et al., 1992) and is associated with an essential enhancer activity in BPV-1 (Vande Pol & Howley, 1990). However mutation of this sequence to GCATTTT (NR2mt2) had no effect on nuclear factor binding in EMSA and multimerized NR2 elements had no observable enhancer activity in CT3 or PalK cells (Fig. 7). In addition, NR2CE2 multimers gave lower enhancer activity than equivalent CE2 multimers in both CT3 and PalK cells (Fig. 7), another indication that NR2 does not possess enhancer activity. Deletion of the entire NR2 element in an earlier study (Jackson & Campo, 1991) led to a five- to sixfold increase in the enhancer activity of a subfragment of the LCR, and whilst the introduction of NR2 mutations 7/6 and 7/8 into the context of the intact LCR did not give an equivalent increase in enhancer activity, the effects of these mutants were consistently reproducible. Indeed for 88529B cells the wild-type BPV-4 LCR had virtually no activity whereas LCR mutants 7/6 and 7/8 gave a significant level of enhancer activity. The NR2 element of BPV-4 thus appears to contribute to down-regulation of the LCR, and may cooperate with other negative factors that also bind the LCR, with the overall LCR activity reflecting the precise combination and quantity of factors in a particular cell type.

Characterization of the NR2-binding factor indicated a relationship with C/EBP. In addition to the initially characterized C/EBPz, several other C/EBP family transcription factors have now been identified including C/EBPβ (LAP, NF-IL6, IL-6DBP, AGP/EBP, CRP2; Akira et al., 1990; Chang et al., 1990; Descombes et al., 1990; Poli et al., 1990; Williams et al., 1991), C/EBPγ (Ig/EBP; Roman et al., 1990) and C/EBPδ (CRP3; Cao et al., 1991; Williams et al., 1991) and these transcription factors bind to DNA both as homodimers and as heterodimers. Although initially identified as an enhancer binding protein, C/EBPz has now been shown to be capable of DNA binding-dependent transcriptional repression and the action of C/EBPz at a particular site may depend either on the specific binding site sequence or on sequence context (Pei & Shih, 1990; Lopez-Cabrera et al., 1990). Regulation of the C/EBP family is highly complex, involving a number of different mechanisms. Alternative translation initiation leads to the production of truncated proteins that attenuate the trans-activation activity of the full-length protein by binding site competition (Descombes & Schibler, 1991; Ossipow et al., 1993). Other nuclear factors can also dimerize with C/EBP family proteins (Ron & Habener, 1992; Vallejo et al., 1993). Phosphorylation of C/EBP transcription factors at various sites has been demonstrated to affect DNA binding (Mahoney et al., 1992) or trans-activation (Trautwein et al., 1993). Thus there are several possibilities that might explain the observed differences in NR2 activity between cell types. We have demonstrated complexes of identical mobility in EMSA using nuclear extracts from PalK, 88529B and CT3 cells, but these complexes may well differ in their subunit composition or phosphorylation status, both of which might affect activity. Although all the complexes from each of the three cell types appeared to contain C/EBPβ, these complexes may also contain other proteins which are able to heterodimerize with C/EBPβ. Alternatively the complexes might be identical in all three cell types with the negative activity mediated by another protein that differs in its expression. In view of the apparent presence of C/EBPβ in the NR2 complexes, it is interesting that Kyo et al. (1993) have recently demonstrated that the expression of exogenous C/EBPβ in HeLa or CaSk cells causes repression of the HPV-16 LCR via C/EBP binding to several sites within the HPV-16 LCR.
The RSV EFII enhancer element which also binds C/EBP transcription factors (Ryden & Beemon, 1989; Sears & Sealy, 1992) contains an inverted repeat motif showing considerable homology to the inverted repeats of NR2, although to align the NR2 inverted repeats with those of EFII a 4 bp gap must be introduced into the NR2 sequence (Fig. 2.d). It is interesting that for both EFII (Sears & Sealy, 1992) and NR2 the 5' repeat was apparently more important than the 3' repeat for nuclear factor binding, although mutations of the NR2 3' repeat did affect binding. The flanking sequence may well influence binding affinity and thus explain the observed difference in nuclear factor binding between the two repeats in each case. Although NR2 and EFII bound similar nuclear factors, NR2 displayed negative activity in some cell types whereas EFII acts as an enhancer in all cells tested. Glass et al. (1988) demonstrated that whilst the thyroid hormone (T₃) receptor trans-activates via a palindromic T₃ response element, it will repress transcription at similar sites with a 3 bp insertion between the two halves of the palindrome. Therefore the authors proposed that the additional bases lead to alterations in the interaction and/or conformation of the T₃ receptor molecules causing a change in activity. It appears possible therefore that while NR2 and EFII bind similar nuclear factors, the conformational constraints imposed by decreasing the spacing of the inverted repeats in NR2 may lead to altered activity. It is also apparent that although EFII is able to compete out all three NR2 oligonucleotide/nuclear factor complexes, some nuclear factors bind to EFII that are not efficiently competed for by NR2 (Fig. 5b), suggesting that the ability to bind these complexes may play an important role in enhancer activity.

Members of the C/EBP family have a highly tissue- or cell type-specific distribution and C/EBP-related nuclear factors have been observed in fibroblasts and epithelial cells (Peterson & Calame, 1987; Roman et al., 1990; Cooper et al., 1992). C/EBPα has been demonstrated to play an important role in the differentiation of adipocytes (Umek et al., 1991) and expression of C/EBPα, -β and -γ is temporally regulated in distinct patterns during differentiation of adipocytes (Cao et al., 1991) and myeloblasts (Scott et al., 1992). It has been proposed that C/EBP may be involved in the differentiation of other tissues and C/EBP has been found in terminally differentiated skin and intestinal cells (Umek et al., 1991). Since transcription and replication of papillomaviruses are tightly linked to epithelial differentiation, it seems possible that C/EBP might be one of the proteins through which this coupling occurs. The activity of the NR2-binding factor does vary between Pa1K and 88529B cells, and we intend to investigate whether a similar change in activity occurs during keratinocyte differentiation. Alternatively, the difference in NR2 activity between the different cell types may relate to the immortalized state of CT3 and 88529B cells.

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References


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