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Differential regulation of different human papilloma virus variants by the POU family transcription factor Brn-3a

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ABSTRACT

The Brn-3a POU family transcription factor is over-expressed in human cervical carcinoma biopsies and is able to activate expression of the human papilloma virus type 16 (HPV-16) upstream regulatory region (URR), which drives the expression of the E6 and E7 oncoproteins. Inhibition of Brn-3a expression in human cervical cancer cells inhibits HPV gene expression and reduces cellular growth and anchorage independence *in vitro* as well as the ability to form tumours *in vivo*. Here we show that Brn-3a differentially regulates different HPV-16 variants that have previously been shown to be associated with different risks of progression to cervical carcinoma. In human cervical material Brn-3a levels correlate directly with HPV E6 levels in individuals infected with a high risk variant of HPV-16 whereas this is not the case for a low risk variant. Moreover, the URRs of high and intermediate risk variants are activated by Brn-3a in transfection assays whereas the URR of a low risk variant is not. The change of one or two bases in a low risk variant URR to their equivalent in a higher risk URR can render the URR responsive to Brn-3a and *vice versa*. These results help explain why the specific interplay between viral and cellular factors necessary for the progression to cervical carcinoma, only occurs in a minority of those infected with HPV-16.

INTRODUCTION

It is now well established that the human papilloma viruses HPV-16 and HPV-18 encode specific transforming proteins (E6, E7) and play a key role in the development of human cervical carcinoma (Durst *et al*., 1983; Schiffman *et al*., 1993). However, HPV-16 and HPV-18 can also be detected in women with no or minimal cervical abnormality (Burghardt *et al*., 1984; Cox *et al*., 1986; De Villiers *et al*., 1987) and it has been shown that in young women HPV infections are usually cleared rapidly and only infrequently lead to cervical transformation (Ho *et al*., 1998). This may be due to the persistence of the virus in some women, whereas it is rapidly cleared by others (Nobbenhuis *et al*., 1999). Alternatively, it has been suggested that progression to disease occurs rapidly in some women and not in others for, as yet unexplained reasons (Miller, 2001; Woodman *et al*., 2001).

These findings suggest that the interplay between viral and cellular regulatory proteins is likely to play a key role in disease progression. Thus, a 100 base pair region of the URR of HPV-16 and HPV-18 has been shown to be specifically active in cervical cells (Cripe *et al*., 1987; Gloss *et al*., 1987) and may thus play a key role in regulating E6 and E7 production. However, the majority of cellular transcription factors which bind to this region are expressed in all cell types and cannot account for its cervical specific activity (Chan *et al*., 1989; Chan *et al*., 1990; Nakshatri *et al*., 1990).

We previously showed however, that the cellular POU family transcription factor Brn-3a (for review of POU factors see Ryan *et al*., 1997; Verrijzer *et al*., 1983) is able to strongly activate transcription via the URR (Morris *et al*., 1994). This is of particular interest since, although Brn-3a was originally identified in neuronal cells (He *et al*., 1989; Lillycrop *et al*., 1992), it has subsequently been shown to be also expressed in cervical cells but not by other cell types (Ndisang *et al*., 1999; Ndisang *et al*., 2000). Moreover, Brn-3a levels are elevated over 300-fold in the cervix of women with cervical intra-epithelial neoplasia type III (CIN3) (Ndisang *et al*., 1998). Elevated Brn-3a levels were observed throughout the cervix in women with CIN3. Hence, over-expression may be dependent upon genetic factors, such as a polymorphism in the Brn-3a promoter or environmental factors, such as smoking, rather than being a consequence of the transformation process itself (Ndisang *et al*., 2000).

These findings suggest that elevated expression of Brn-3a in particular women may act to activate HPV gene expression thereby producing cervical carcinoma in the regions where HPV is located. In agreement with this critical role for Brn-3a, we have shown that reducing the level of Brn-3a in a human HPV-transformed cervical cell line results in reduced expression of the HPV E6 and E7 proteins. Moreover, these cells also show reduced growth rate saturation density, ability to grow in an anchorage independent manner in soft agar (Ndisang *et al*., 1999) and most importantly, reduced ability to form tumours *in vivo* in nude mice (Ndisang *et al*., 2001).

These findings indicate therefore that the over-expression of Brn-3a in human cervical carcinoma is paralleled by its ability to activate HPV gene expression and to alter cellular growth rate both *in vitro* and *in vivo*. Evidently, to achieve its effect Brn-3a must interact with HPV-16 or HPV-18 to stimulate their transforming activity. However, a number of different variants of HPV-16 and HPV-18 have been identified which show specific sequence alterations in the URR that correlate with their transforming ability (Kämmer *et al*., 2000; Villa *et al*., 2000). Similarly, sequence variants in the E5 region of HPV-16 that are associated with differences in viral transcription levels and induction of neoplasia have been previously defined (Bible *et al*., 2000). This study is of particular interest since it compared HPV variants in infected women with no clinical abnormality with those found in women with cervical neoplasia.

In this study, we have therefore examined the effect of Brn-3a on the different HPV variants which were defined by Bible *et al*., (Bible *et al*., 2000) on the basis of differences in the E5 region in order to determine whether their different viral transcription levels and ability to induce neoplasia are related to their response to Brn-3a, as well as to define the molecular basis for any such differences in their responses.

MATERIALS AND METHODS

Cervical samples

The study group consisted of 86 women who were referred to the Colposcopy Clinic at the Whittington Hospital, London, U.K., for investigation of an abnormal smear and 16 women attending gynaecology outpatient clinics at Guy's and St Thomas' Hospital London, U.K. The women provided informed consent at the time of colposcopy. Ethical permission was obtained from the Hospital's Ethics Committee after review of the study protocol. The mean age of the study group was 33.71 years (median 30 years, range 17-89 years). The reference smears of the Whittington patients were classified as Borderline 33, Mild Dyskaryosis 21, Moderate Dyskaryosis 20, Severe Dyskaryosis 8, Dyskaryosis difficult to grade 4. Moreover, 3 of the cytological samples from Guy's and St Thomas' Centre were severe dyskaryosis, 7 were moderate dyskaryosis, 5 were mild dyskaryosis and 1 was borderline. From the 102 women that formed the study group, 52 of them were smokers, 45 were non-smokers and 5 were ex-smokers. The smokers were smoking on average 8 cigarettes per day. 88 women were pre-menopausal, 12 menopausal and 2 peri-menopausal.

Measurement of Brn-3a and HPV E6 levels in cervical material

Brn-3a and HPV E6 levels were determined using a previously described RT-PCR assay (Ndisang *et al*., 1998). Routinely about 0.1 μg of DNase treated RNA extracted from cervical material, obtained by colposcopy was used as a template for cDNA synthesis. Equal amounts of the resultant cDNA were then used for RT-PCR. In brief, respective aliquots of the synthesised cDNA, were sequentially amplified through 15, 20 and 25 thermocycles using Brn-3a, HPV E6 and cyclophilin specific primers (this was to ensure that the amplified product was quantitatively related to input cDNA over this range of cycles). In all quantitative assay cases, Brn-3a and HPV E6 were amplified for 25 cycles, whilst cyclophilin products were subjected to 17 cycles. These conditions were shown to be within the range of proportionality of the PCR reaction in which the signal is directly related to the amount of template present.

Amplicons were fractionated on a 2% agarose gel. The gel was blotted onto Hybond-N+ nylon membrane (Amersham), and hybridized with homogenous complimentary ^{32}P-labeled probes. Membranes were used to expose Kodak films and the resultant autoradiograph analysed using a densitometer with Molecular Analyst software (BioRad, Labs). The densitometric value of the negative control in all instances was negligible. This minimum background was subtracted from the respective samples. Thus the levels of Brn-3a and E6 amplicons was compared to that of the constitutively expressed cyclophilin in each of the samples.

Generation of E6 URR variant Luciferase constructs

E6 promoter fragments were amplified from cervical smears infected with HPV16 variant 1, variant 2 and variant 5 DNA using primers ‘E6-upstream-Xho’ (5’ CCC-TCG-AGC-TGT-AAG-TAT-TGT-ATG-TAT-GT 3’) and ‘E6-downstream-NcoI’ (5’ CCC-CAT-GGC-AGT-TCT-CTT-TTG-GTG-CAT-A), designed using the published HPV16 sequence (GenBank accession number NC_001526). The annealing temperature used was 65^{o}C. The resultant products being the full 5’ flanking region of the E6 gene from the final codon of L1 (the upstream coding sequence) to the seventh codon of the E6 coding sequence.

The amplified products were cloned into the pGL3-Basic Luciferase reporter vector (Promega) using restriction sites XhoI and NcoI (present in both vector and PCR primer flanks). The resulting clones were sequenced in both directions (MWG-Biotech) to confirm

insertion in frame, and to identify sequence differences between variants. Constructs were termed V1, V2 and V5 and confirmed by sequence analysis.

Generation of E6 URR point mutation constructs

To determine to what extent the sequence differences between V1 and V5 contributed to the change in sensitivity to Brn3a induction, we used site directed mutagenesis ("QuikChange", Stratagene) to change specific bases. At positions M1, M3 and M4 (see Fig.3) of V1 or V5, bases were independently mutated to the sequence of the other variant. Correct mutation of sequences were confirmed by sequence analysis. The nomenclature used for the resultant clones denotes whether the clones had variant 1 sequence (1), or variant 5 sequence (5) at each of these positions.

Generation of further E6 URR substitution constructs

To generate further Luciferase reporter constructs, restriction enzymes were used to switch reciprocal promoter regions. In constructs 11511 and 55155 Eco72I and EcoRI restricted fragments were exchanged to generate 15111 and 51555. Using the same constructs (11511 and 55155) SmaI/EcoRI fragments were exchanged to generate 11555 and 55111. Similarly wild-type variant 1 and variant 5 (11111 and 55555) were subjected to SmaI/EcoRI substitution to yield 11155, and 55511. Finally, constructs 11151 and 55515 were subjected to EcoRI/Nco1 fragment exchange giving constructs 11115 and 55551. All constructs were tested in the transfection system described below.

Transfection and reporter gene assay

Transfections

SiHa cells (ATCC) were routinely grown in minimum essential medium (Eagle's) with Earle's buffered saline solution, supplemented with 10% fetal calf serum, 0.1mM nonessential amino acids, and 1.0mM sodium pyruvate. The SiHa cells were transfected by the calcium phosphate method as before (Ndisang *et al*., 1999). In brief, 1μg of the HPV URR recombinant pGL3 reporter plasmid was co-transfected with 1μg of the expression vector pLTRpoly (ATCC) containing the full-length cDNA of Brn-3a transcription factor under the Moloney murine leukaemia virus promoter (Ndisang *et al*., 1999; Ndisang *et al*., 2001), and 0.1 μg of TK-Renilla plasmid (Promega) as an internal control onto $0.7x10^5$ SiHa cells. The cells were harvested after 48 hours and washed with 1 x PBS.

Luciferase assay of reporter gene activity

Transfected cells were lysed in 1 x passive lysis buffer (Promega) and assayed for both firefly and Renilla luciferase activities according to the manufacturer's protocol, with results measured and quantified on a TD-20e Luminometer. The Relative Luciferase Units (RLU) which is the ratio of the firefly's activity to that of the Renilla was then obtained. Each transfection was done in triplicate and thus, the mean and standard deviations were determined.

DNA mobility shift assay

Analysis of DNA-protein interactions was performed generally as previously described (Vivanco *et al*., 1995). Briefly, GST proteins were purified from *E. coli* as described previously (Gascoyne *et al*., 2004) and doubled-stranded oligonucleotides

radioactively labelled using T4 polynucleotide kinase. 15fmol oligonucleotide (approx. 5000 cpm) was incubated with 50ng purified protein in binding buffer (20 mM Hepes-KOH pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 15 % glycerol, 1 mM DTT, 5 ng/µl polydIdC, 10mM herring sperm DNA) for 20 minutes at 20-25^{o}C in each case. Pre-incubations with specific and non-specific oligonucleotides were performed for 5 minutes prior to addition of labelled oligonucleotide. Oligonucleotides (Thermo-Electron, Germany) used were as follows:

Consensus; 5'-CTCCTGCATAATTAATTACGCCCG-3',

Non-specific; 5'-CCCTTACATACCGCTGTTAGGCACATATG-3',

SiteM3(V1); 5'-CGTATATAAACTATATTTGCTACATCCTG-3',

SiteM3(V5); 5'-CGTATATAAACTATATTTGCTACAATCTG-3',

SiteM4(V1); 5'-CTGTCAGCAACTATGGTTTAAACTTGTAG-3',

SiteM4(V5); 5'-CTGTCAGCAACTATAGTTTAAACTTGTAG-3',

SiteM5(V1); 5'-CACTAAACTACAATAATTCATGTATAAAG-3',

SiteM5(V5); 5'-CACTAAACTACAATAATCCATGTATAAAG-3'.

Chromatin immunoprecipitation assay

This technique was carried out using our previously described methodology (Lee *et al.,* 2005). Briefly, cross-linking was done by addition of 270 µl of 35% formaldehyde to 10ml of medium and incubation for 15 minutes. The SiHa cells were washed, harvested in cold PBS, and then lysed in lysis buffer [(1% SDS, 0.01 mol/L EDTA, 0.05mol/L Tris (pH 8.0), and 0.01% protease inhibitor cocktail)]. The cells were sonicated and then centrifuged. A small volume of the supernatant was taken out for use as input sample in subsequent PCR analysis, while the remaining sample was divided and then subjected to immunoprecipitation overnight using the appropriate antibody, either Brn-3a mouse monoclonal antibody (Chemicon) or secondary anti-mouse antibody (DAKO). Incubation with protein G-Sepharose beads allowed immobilization of protein-antibody complex that was washed thoroughly and then eluted from the beads. The cross-links between the protein and the DNA were reversed by incubation at 65^0 C for 4 hours and proteins were digested using proteinase K. Following phenol/chloroform extraction, the DNA was precipitated with ethanol, resuspended in water, and used for PCR. The primers used to amplify the URR region containing the Brn-3 site were; HPVF 5'-GAA TCA CTA TGT ACA TTG TGT C-3' and HPVR 5'-ACC TTT ACA CAG TTC ATG TAT-3' using the following thermal cycle: 1 cycle at 94^0 C for 15 minutes followed by 40 cycles of 95^0 C for 30 seconds, 58^0 C for 30 seconds, and 72^0 C for 30 seconds, and with a final incubation at 72^0 C for 5 minutes. The amplicons were then resolved on a 2.5% agarose/Tris-borate EDTA gel.

RESULTS

To evaluate the effect of Brn-3a on the different HPV-16 variants, we utilised a reverse transcriptase-polymerase chain reaction (RT-PCR) assay which we have previously used to successfully measure Brn-3a and HPV E6 mRNA expression in cervical biopsies and cervical smears (Ndisang *et al*., 1998; Ndisang *et al*., 2000; Sindos *et al*., 2003) with Brn-3a mRNA levels correlating well with Brn-3a protein levels when sufficient material is available for these to be measured (Ndisang *et al*., 1998). The levels of Brn-3a and HPV E6 mRNA were measured in a series of colposcopy samples which had previously been typed according to their variation in the region of the HPV-16 genome encoding E5 (Bible *et al*., 2000). As expected, three major variants were present in the different human cervical samples. These have been previously defined as variant 5 (17% prevalence) which is negatively associated with viral transcription and cervical neoplasia in HPV-infected women; (odds ratio 0.2) variant 2 (50% prevalence) which is positively associated with disease; (odds ratio 2.57) and variant 1 (24% prevalence) which shows an intermediate risk of disease (Bible *et al*., 2000).

In this study, we observed a very clear correlation between Brn-3a mRNA levels and HPV E6 levels in samples from women infected with the high risk variant 2 (Fig.1). In contrast, no HPV E6 mRNA was detectable in women infected with variant 5 regardless of the Brn-3a mRNA level. In women infected with variant 1 which is of intermediate risk, E6 mRNA was detectable. However, whilst there was a correlation with Brn-3a, it was less strong than that observed for variant 2. Similar results were also obtained when real time PCR was used to quantitate the levels of the different mRNAs using the QuantiTect SYBR green PCR kit (Quiagen, Crawley, U.K.) and an Opticon ^{TM} DNA engine (BioRad Hertfordshire, U.K.) thermal cycler (data not shown) as in our previous work quantitating Brn-3a (Hudson *et al*., 2004; Ripley *et al*., 2005). Thus, HPV E6 mRNA was detectable at low levels in the variant 5 samples by real time PCR but the levels in the variant 2 samples were approximately ten-fold higher (data not shown).

Hence, these results indicate that the high risk variant 2 shows significant transcription of the HPV E6 gene which correlates with the expression of the transcriptional activator Brn-3a, whereas no significant HPV E6 mRNA expression is observed from variant 5, regardless of the level of Brn-3a.

To determine whether these differences reflected differences in the responsiveness of the URR of each variant to Brn-3a, the URR of at least two different isolates of each variant was cloned upstream of a luciferase reporter gene and transfected with a Brn-3a expression vector into the human SiHa cervical carcinoma cell line. Although the URR of variant 5 provided detectable basal promoter activity above that observed with the control empty vector, it was not activated by Brn-3a whereas strong activation was observed for the URRs derived from variant 2 isolates with activation also being observed with variant 1 but to a slightly lower extent (Fig.2a). Similar effects were also observed in BHK fibroblast cells transfected with the URR constructs and Brn-3a indicating that this effect is not specific to HPV-expressing cervical cells. In all transfection experiments equal levels of Brn-3a were observed by western blotting in the Brn-3a transfected cells, regardless of the co-transfected HPV variant (see for example Fig.2b).

Therefore, the differences in HPV mRNA expression relative to Brn-3a expression in cervical samples infected with different variants are paralleled by differences in their responsiveness of the URRs derived from these different variants. Hence, the variation in E5 sequences, which was used initially to define the different variants, appears to be associated with variations in their respective URRs. In turn, these variations give rise to differences in the response to Brn-3a and hence in the level of mRNA encoding E6 which is observed in each case for a given level of Brn-3a expression. In turn, since HPV E6 is a transforming

protein, these differences in the URRs may underlie not only the previously defined differences in viral transcription but also the differences in transformation by each variant.

In view of the critical role of this difference in the URR response to Brn-3a in each different variant, we investigated the molecular basis of this difference. To do this we characterised the URR DNA sequences, again using two different isolates of each variant. As indicated in Fig.3, the three variants are very similar to one another with only variation in four single nucleotides and one dinucleotide as well as a 38-base pair deletion in variant 2 compared to variants 1 and 5.

To eliminate any complications caused by this deletion in variant 2, we chose initially to compare the Brn-3a-inducible variant 1 and the non-inducible variant 5, which differed only at five positions (four point mutations and one dinucleotide change). Each of these positions was individually altered in each variant to the equivalent in the other variant by a combination of site directed mutagenesis and exchange of restriction enzyme fragments. Each of these variants was then tested for its response to Brn-3a by transient transfection.

In these experiments, several of the mutants showed inducibility by Brn-3a which was comparable to that of variant 1 whereas others showed the lack of inducibility characteristic of variant 5 (Fig.4). In particular, strong inducibility was shown by mutants 55515 and 55155 which have a backbone of variant 5 but respectively have either a single A to G change at variation site 4 or AT to TC change at variation site 3 (see Fig.3). Conversely, mutants 11151 and 11511 which have the reciprocal variations in a variant 1 background have lost the inducibility characteristic of variant 1. In contrast, mutation at position 2 or position 5 conferred minimal inducibility on a variant 5 backbone (mutant 51555 or mutant 55551), although in each case it did abolish inducibility in a variant 1 backbone (mutant 15111 and mutant 11115). Meanwhile exchange at position 1 had no significant effect on inducibility (mutants 15555 and 51111). Hence, specific one-base or two base changes can confer inducibility on an otherwise non-inducible HPV variant or abolish the inducibility of an otherwise inducible variant.

To extend these experiments, we included the strongly inducible variant 2 in our analysis. The TC at position three in variant 2 was altered to the AT found in variant 5 whilst independently, the A at position four was changed to the G found in variant 1. Each of these changes resulted in the loss of inducibility by Brn-3a (Fig.5). Hence, both in variants 1 and 5 which contain an extra 38 base pair sequence and in variant 2 which lacks it, the precise sequence at sites 3 and 4 determines inducibility by Brn-3a.

To determine whether these sites achieved their effect by directly binding Brn-3a, we carried out DNA mobility shift assays using bacterially-expressed Brn-3a. In these experiments only weak or no binding of Brn-3a was observed with oligonucleotides containing either of the variant sequences found at positions 3 or 4 (Fig.6). This suggests that these sites do not exert their effect by direct binding of Brn-3a. Rather, the sequence differences at these sites are likely to affect the binding of other transcription factors which then interact with Brn-3a bound to conserved and/or variant sites elsewhere in the URR. Interestingly, an oligonucleotide containing the sequence found at position 5 in variant 1 did bind Brn-3a and this binding was reduced by the sequence change observed in this site in variant 5. This indicates therefore, that position 5 represents one site for Brn-3a binding in the URR which can influence its effect on URR activity.

To confirm that Brn-3a binds to the HPV URR in intact cells, we carried out a chromatin immunoprecipitation (ChIP) assay using SiHa cells which have endogenous HPV genomes. HPV URR DNA was clearly amplified from the sample of SiHa chromatin immunoprecipitated with Brn-3a antibody compared to the background level in the control

sample (Fig.7). Hence, Brn-3a does indeed bind to the URR within the endogenous HPV genome, in intact SiHa cells.

DISCUSSION

In our previous work, we have demonstrated that the Brn-3a POU family transcription factor plays a key role in the ability of HPV-16 to transform cervical cells. In the work described here, we have related these effects of the cellular Brn-3a transcription factor to previously described reports that different variants of HPV-16 produce different risks of disease progression following initial infection. Thus, the HPV variants of Bible *et al*., (Bible *et al*., 2000) which were shown to be associated with different risks of progression to cervical carcinoma have been shown to respond differently to Brn-3a both in transfection experiments in cultured cells and in cervical smears taken from women with cervical neoplasia. Thus, HPV-16 variant 2, which shows a high risk of disease progression shows a strong correlation of HPV E6 mRNA levels with Brn-3a mRNA levels in human cervical smears, whereas this is not observed with the low risk variant 5. Thus, the differences in risk between the different variants previously described by Bible *et al*., (2000) correlate with responsiveness of the URR to activation of its expression by Brn-3a.

These differences in responsiveness to Brn-3a inferred from the clinical material can be directly demonstrated in transfections of human cervical cells. Thus, the URR promoter sequence of variants 1 and 2 is activated by Brn-3a whereas no such activation is seen in variant 5. As well as extending our results to the molecular level, these results suggest that the variation in the coding sequence for the HPV E5 protein which was originally used by Bible *et al*., (Bible *et al*., 2000) to characterise the different variants may represent a marker for the variation in URR sequence between the different variants which determines the differences in their response to Brn-3a and hence the risk of disease progression.

In particular, sequences comparisons of the different variants revealed four single-base pair changes and a dinucleotide change as well as a 38-base pair deletion in variant 2 which was retained in variants 1 and 5. By altering each of the single or dinucleotide between variant 1 and variant 5 to the sequence found in the other variant, we were able to analyse the reasons for their different responsiveness to Brn-3a. In particular, alteration of a single A residue at position 4 in variant 5 to the G residue found in variant 1 was able to produce a Brn-3a inducible URR, whereas the converse change abolished this effect. Similarly, change in the dinucleotide AT found at position 3 in variant 5 to the TC found in variant 1 again produced an inducible variant 5 differing by only two bases from the original.

This two-base substitution at position 3 is of particular interest since both variant 1 and the high-risk variant 2 have TC at this position, whereas the low-risk variant 5 has AT. Indeed, mutation of this TC to AT in variant 2 abolished its inducibility by Brn-3a confirming the key role of this site. Interestingly however, at position four, variant 2 resembles variant 5 in having an A residue whereas variant 1 has a G. Once again, mutation of the A residue in variant 2 to a G residue abolished its inducibility by Brn-3a. Hence, it is likely that the combination of different bases at each of the variant positions is likely to determine the inducibility of a particular URR by Brn-3a. However, in all three variants, the residues at positions 3 and 4 play a key role in determining their inducibility, operating in the context of the entire promoter sequence of each variant.

In this regard, it is of particular interest that neither of the variants of position 3 and 4 bind Brn-3a strongly in DNA mobility shift assays (Fig.6). However, computer sequence analysis of these sites suggests that position 3 may bind the c-ETS-1 transcription factor in variants 1 and 2 only whilst position 4 may bind the promyelocytic leukaemia zing finger protein and paired domain proteins in variants 2 and 5. Hence, proteins whose binding to these sites varies in the different variants may interact with Brn-3a and other factors bound to common or variant sites in the different URR variants to determine the inducibility of each variant. In agreement with this possibility, we have previously shown that Brn-3a binds to a

non-polymorphic site between -255 and -248 in the HPV-16 URR, bases 7730-8 in Fig.3, (Dent *et al*., 1991).

Moreover, we did observe strong binding of Brn-3a to the sequence at position 5 found in variant 1 with weaker binding being observed to the sequence found in variant 5 (Fig.6). In this case it is particularly noteworthy that conversion of this site in variant 1 to its equivalent in variant 5 renders the URR non-inducible (Fig.4), although the converse change does not render variant 5 inducible. This suggests that the effects we are analysing are complex but do involve Brn-3a binding to the URR and interacting with other proteins bound elsewhere, with the precise combination of various binding sites determining inducibility. Hence, several different cellular transcription factors including Brn-3a are likely to interact with one another to determine the level of URR-driven gene expression.

Whatever the case, it is clear that very limited sequence differences between different HPV variants can have a dramatic effect on their inducibility by Brn-3a and therefore, on the level of expression of the E6/E7 oncogenes. Hence, the risk of disease progression following infection by HPV-16 involves both the levels of the cellular transcription factor Brn-3a and the precise variant of HPV-16 with which the woman is infected, with these two factors interacting with one another via the differential responses of different HPV-16 variants to activation by the cellular Brn-3a factor. Taken together, these differences in cellular and viral factors are likely to underlie the previously observed differences in response to HPV infection with some women clearing the virus rapidly whilst in others the disease progresses to cervical transformation (Ho *et al*., 1998; Nobbenhuis *et al*., 1999; Miller, 2001; Woodman *et al*., 2001).

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FIGURE LEGENDS

Figure 1

Levels of HPV E6 mRNA and Brn-3a mRNA, as determined, by reverse transcriptase-polymerase chain reaction assay in cervical smears from women infected with the different HPV-16 variants 1, 2 and 5. Panel a) shows a typical result with samples from HPV-16 variant 2 (tracks 3 and 4) and variant 5 (tracks 1 and 2). Cyclophilin mRNA levels were recorded to allow normalisation of the data. Panel b) shows the normalised data for all samples tested. Note the strong correlation between Brn-3a mRNA and HPV E6 mRNA levels in women infected with the high risk variant 2, the weaker correlation in women infected with the intermediate risk variant 1 and the lack of response to Brn-3a in women infected with the low risk variant 5.

Figure 2

(a) Luciferase activity assay in the human cervical cell line SiHa transfected with constructs in which the upstream regulatory region (URR) of variant 1, variant 2 or variant 5 drives the expression of the luciferase reporter gene. Reporters were co-transfected with either a Brn-3a expression vector (Brn-3a) or control (the same vector lacking any insert, LTR, set at 100%). Values are the mean of six experiments whose standard deviation is shown by the bars. Note the response of the URRs of variants 1 and 2 to Brn-3a and the lack of response of variant 5.

(b) Western blot to measure Brn-3a levels in SiHa cells transfected with Brn-3a expression vector and either URR variant 5 (track 1), variant 2 (track 2) or variant 1 (track 3) or the vector lacking any insert (track 4). Samples were equalised on the basis of the activity of a co-transfected plasmid in which the thymidine kinase promoter drives the Renilla luciferase gene.

Figure 3

Sequence analysis of the URR of the different HPV-16 variants as determined from two independent isolates of each variant, variant 1 sequence is shown in full with the numbering corresponding to the published sequence (NC_001526). Differences between the three variants shown below. Note that the published sequence has GGCCC at 7432-36 whereas all our products had GCGGCC (one extra base), and GAT at 7860-2 whereas all our sequences had GT (lacking one base). Key restriction enzyme sites are single underlined. These are, in order, EcoR72I, EcoRI, PmeI, PmeI, AgeI. The five points of variation are marked M1-M5 and highlighted in bold type, as is the insertion in variants 1 and 5 with respect to variant 2. *The E6 gene reads through the end of the sequence (7904 as published) thus ends at base 85 at the beginning of the entry. TAA in bold at 7152-4 is the stop codon of the L1 gene. Note the L1 poly A signal at 7257-65 and the E6 TATA boxes at 17-23 and 65–71 which are all double-underlined. The previously characterised Brn-3a binding site is AATTGCAT at positions 7730-8. The diagnostic restriction sites in the HPV E5 sequence which were used to define these variants (Bible *et al*., 2000) are also indicated.

Figure 4

Luciferase assay of the various mutant constructs in which individual bases (or two adjacent bases)in one URR variant have been changed to their equivalents in another variant. All values shown are the induction in activity in response to co-transfection with the Brn-3a expression vector Brn3a-LTR. Values are recorded as a percentage of that seen following co-transfection with the empty expression vector alone (LTR) set at 100. In each case the five

sites of variation (shown as M1-M5 in Figure 3) are shown as either having the sequence found in variant 1 or that found in variant 5.The number 1 indicates that the URR contains the base characteristic of variant 1 at each position, whereas '5' indicates that it contains the base characteristic of variant 5 at this position. Thus 11111 denotes wild-type variant 1, whereas 55155 denotes variant 5 with TC (the variant 1 sequence) at site M3. Values are the mean of three experiments whose standard deviation is shown by the bars. Note the significant effect in particular, of alterations at positions M3 or M4 where a single alteration can render a variant 1 backbone non-inducible by Brn-3a and correspondingly render a variant 5 backbone inducible by Brn-3a.

Figure 5

Luciferase assay indicating the effect of altering the bases in variant 2 at position 3 (V2-M3) or position 4 (V2-M4) to their equivalents in variants 5 and 1 respectively. Values are the mean of three experiments whose standard deviation is shown by the bars. Note the loss of inducibility in each case when the bases in the normally inducible variant 2 are changed. Values shown are the induction in activity in response to co-transfection with the Brn-3a expression vector Brn3a-LTR, relative to the level of expression in the presence of the empty expression vector (LTR) which is set at 100.

Figure 6

(a) EMSA analysis of GST-Brn-3a POU binding to putative Brn-3a recognition sequences. Equal amounts of oligonucleotides containing sites M3, M4 or M5 from the V1 URR or a Brn-3 consensus were radioactively labelled and incubated with purified GST (G) or GST-Brn-3aPOU (3). Competition to assess specificity of binding was performed using unlabelled oligonucleotides: "n" indicates presence of 50-fold molar excess of non-specific oligonucleotide, "s" indicates 50-fold excess of specific competitor, same as labelled oligonucleotide.

(b) EMSA analysis of GST-Brn-3a POU binding to labelled consensus Brn-3a element. Binding analyses were performed in the presence or absence of 50-fold or 10-fold competitor unlabelled oligonucleotides containing putative binding sites as shown. All oligonucleotides are as per V1 sequence, except where underlined (V5 sequence).

Figure 7

Chromatin immunoprecipitation (ChIP) assay amplifying the HPV URR from SiHa chromatin (S). Track I shows the input to the immunoprecipitation whereas track - shows the sample immunoprecipitated with the secondary antibody alone and track + shows the sample immunoprecipitated with anti-Brn-3a antibody. The track labelled W is a control PCR with water replacing the chromatin sample.

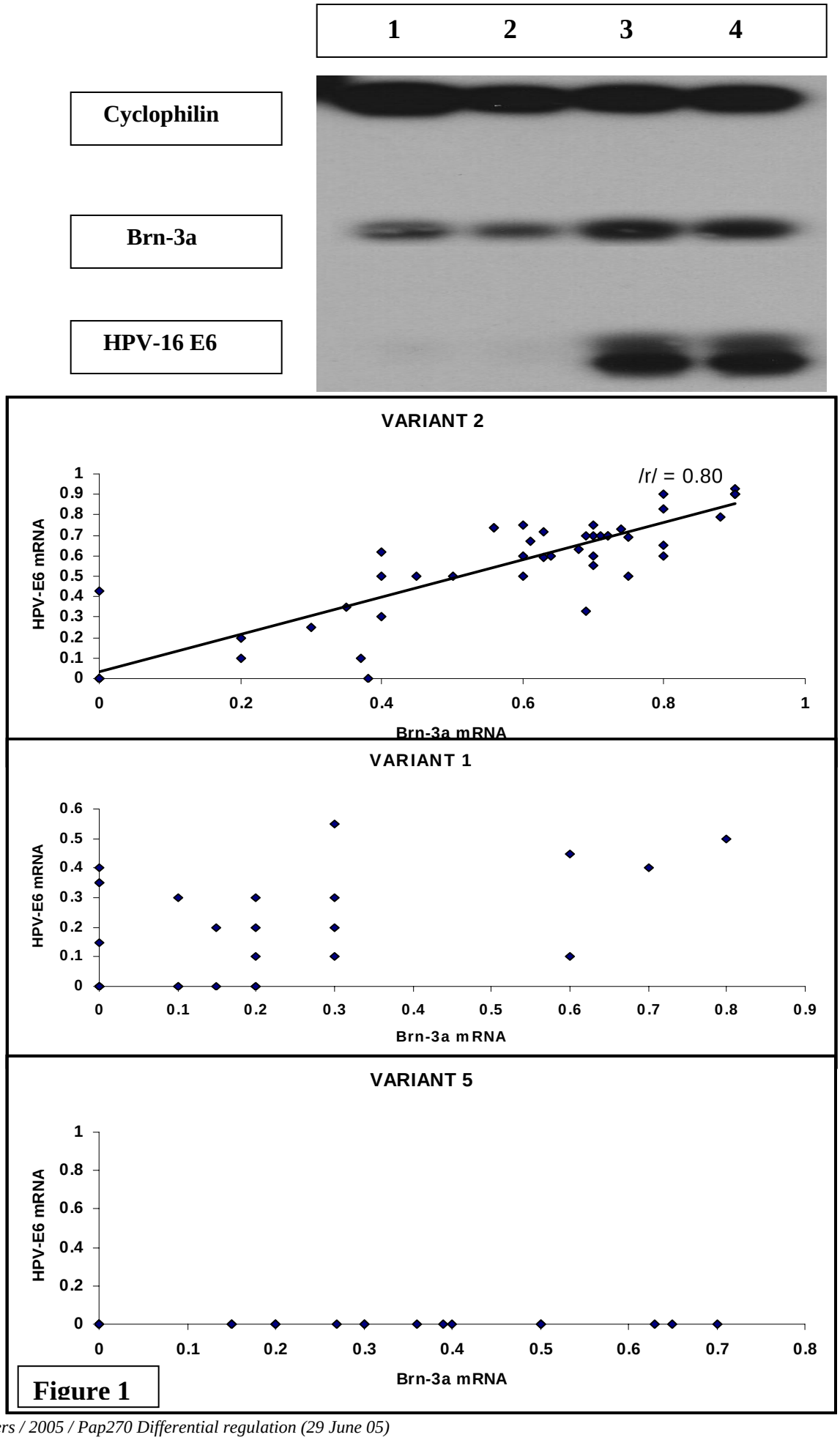

1
2
3
4
Cyclophilin
Brn-3a
HPV-16 E6
VARIANT 2
/r/ = 0.80
HPV-E6 mRNA
Brn-3a mRNA
VARIANT 1
HPV-E6 mRNA
Brn-3a mRNA
VARIANT 5
HPV-E6 mRNA
Brn-3a mRNA

Figure 1

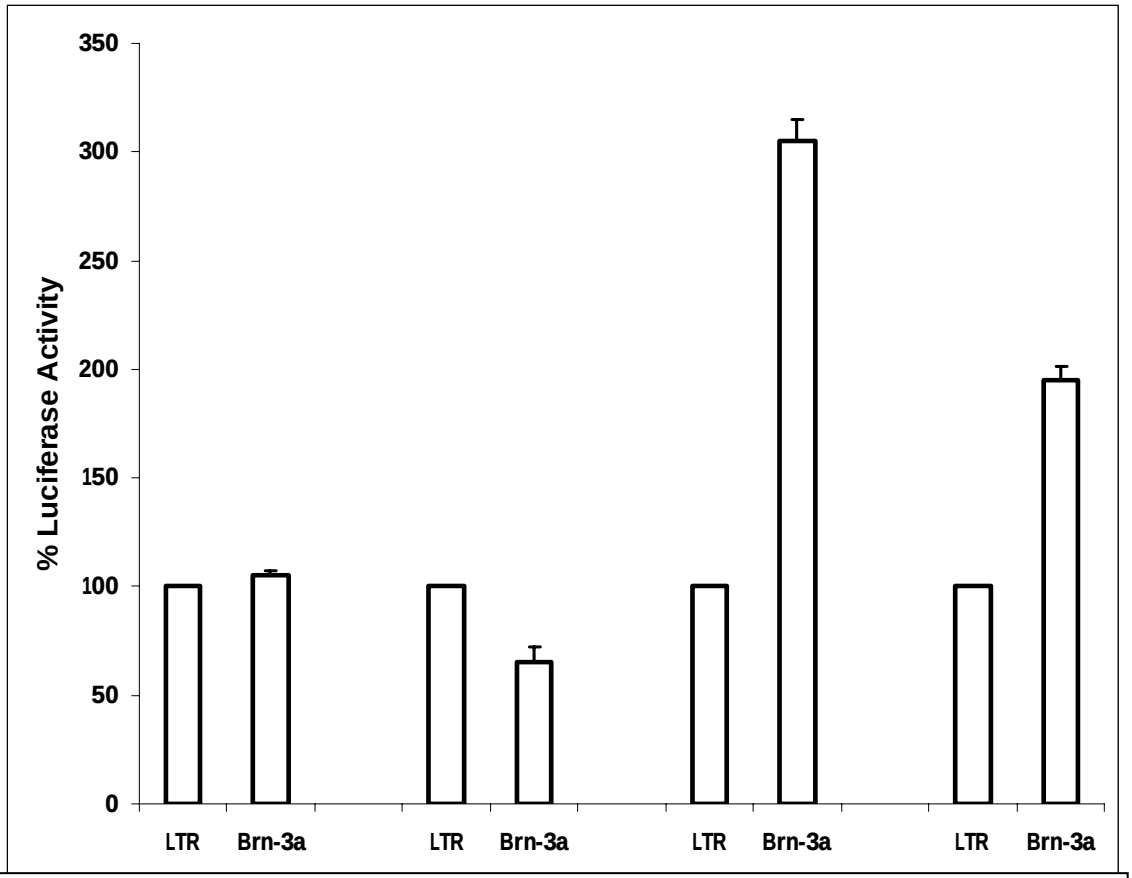


Figure 2a

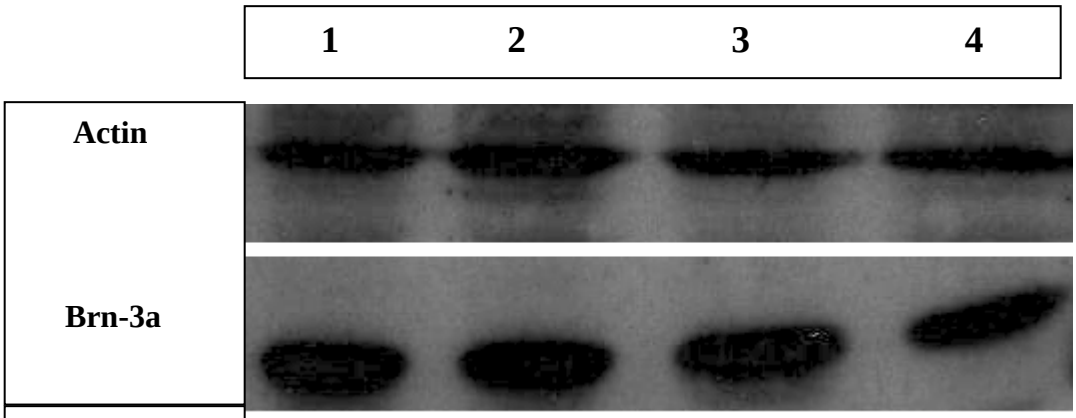


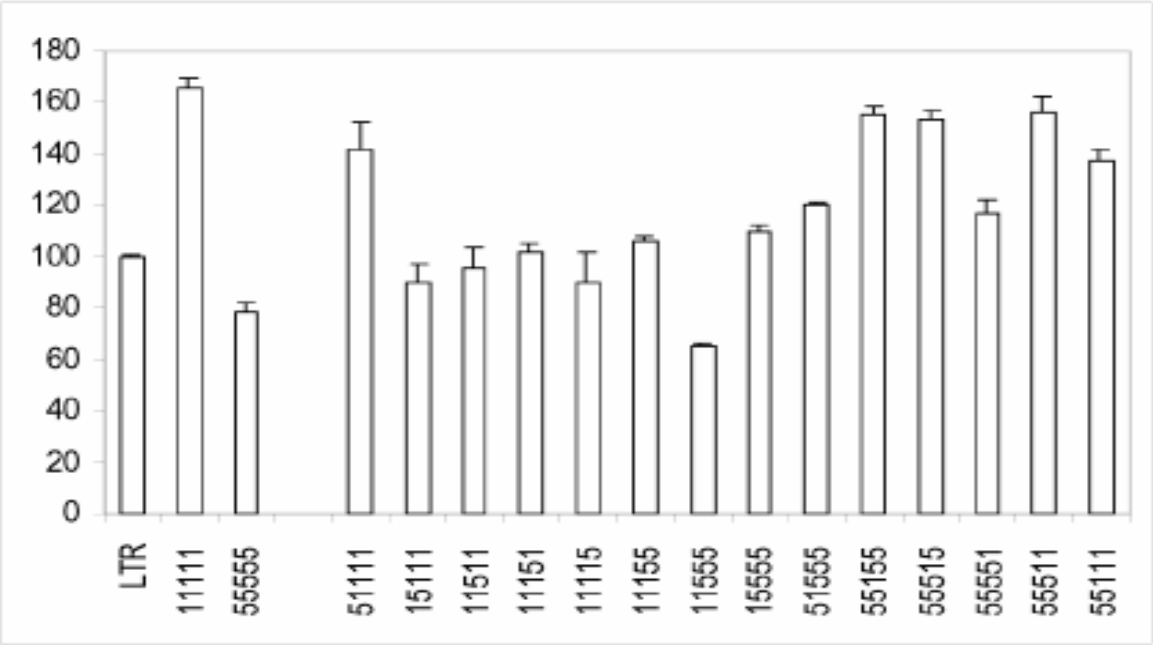
Figure 2b

Figure 3

	URR						E5	
	M1	**M2**	**M3**	**M4**	**Insertion**	**M5**	**Ssp1**	**Xcm1**
NC_001526	G	C	TC	G	Present	T	+	+
Variant 1	G	C	TC	G	Present	T	+	+
Variant 2	T	C	TC	A	Absent	C	-	+
Variant 5	T	G	AT	A	Present	C	-	-

Figure 3

Figure 4


180
160
140
120
100
80
60
40
20
0
LTR
11111
55555
51111
15111
11511
11151
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55551
55511
55111

Figure 5

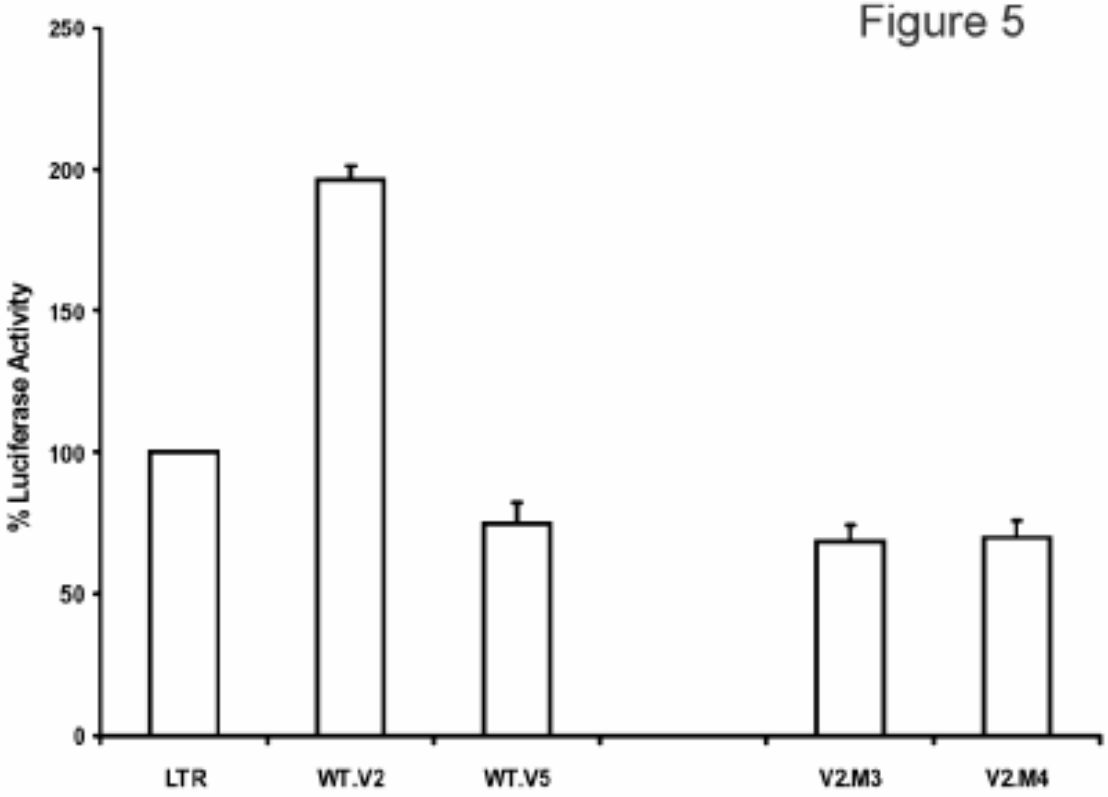
250
200
150
100
50
0
% Luciferase Activity
LTR
WT.V2
WT.V5
V2.M3
V2.M4

Figure 6a

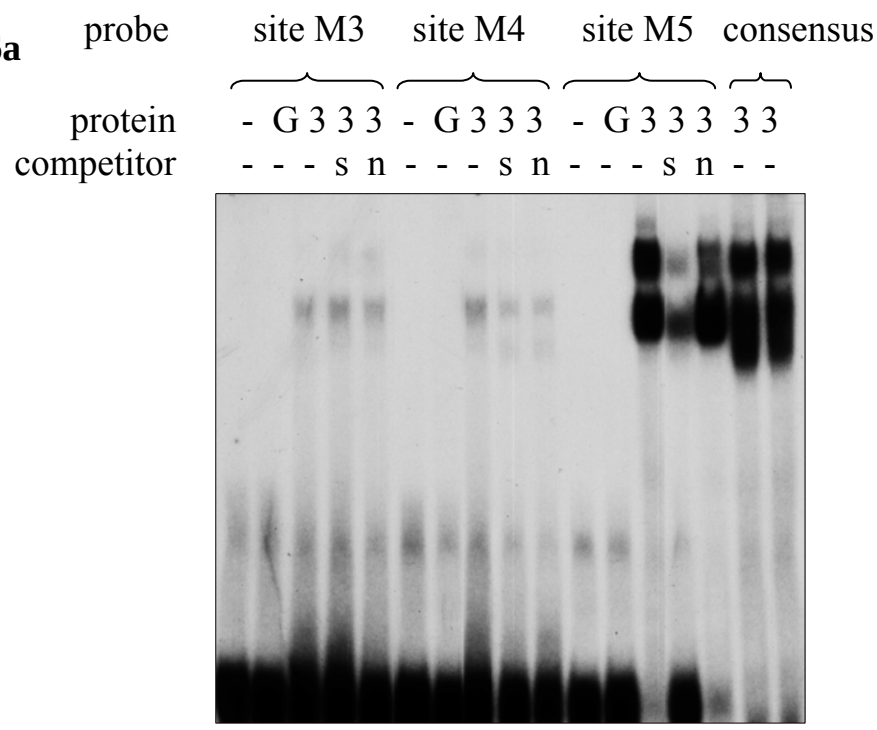

Figure 6b

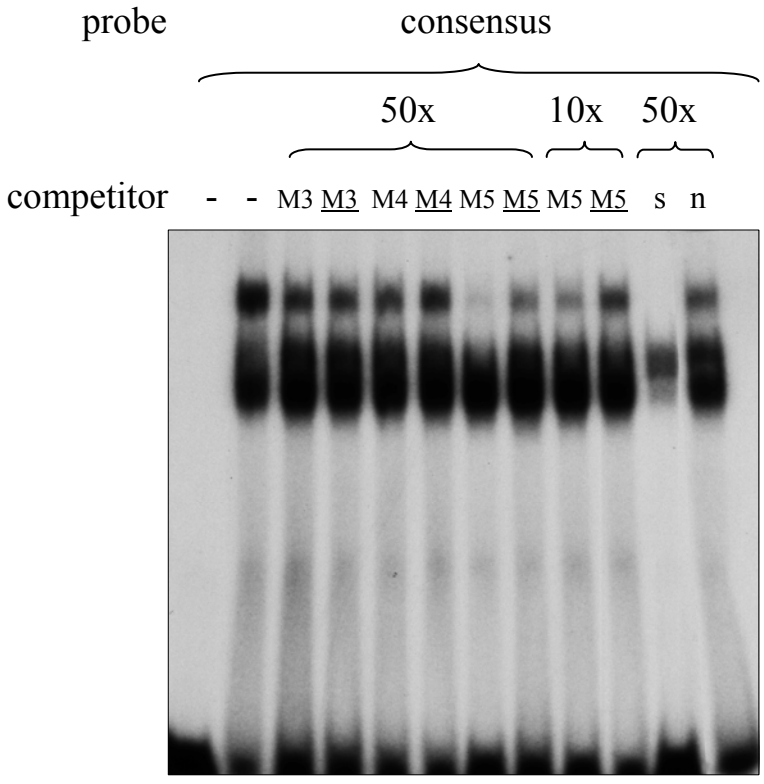

Figure 6

S	S	S	W
I	-	+	

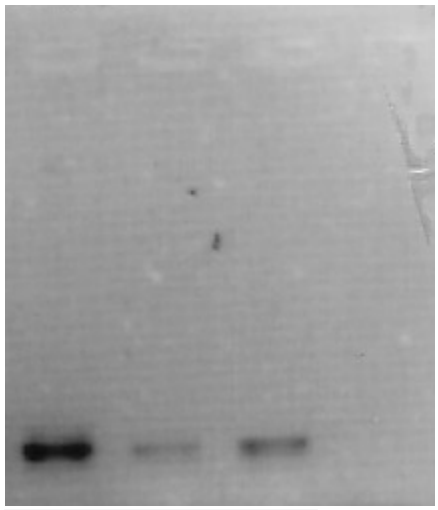

Figure 7.0