Regulation of human papillomavirus type 16 DNA replication by E2, glucocorticoid hormone and epidermal growth factor

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The E1 and E2 proteins are the only human papillomavirus (HPV) proteins required for transient replication of plasmids containing the viral origin. The E2 gene products play key roles in both viral transcription and replication. In this study we have analysed in further detail the nature of the association between E1 and E2 using a series of E2 proteins mutated in conserved regions of the N-terminal domain. These proteins were tested for their ability to activate transcription and to stimulate viral DNA replication. Several of these mutants revealed that the two functions of E2 can be separated, and that they define three widely spaced regions of the N-terminal domain which are important for DNA replication, two of which retain E1-binding activity. This suggests that E2 may have a role in viral DNA replication other than simply localizing E1 to the origin of replication. Additional important elements for regulating viral gene expression have been shown to be glucocorticoid hormones and epidermal growth factor (EGF). We show here that they may also be involved in regulating viral DNA replication. Our studies show that the addition of glucocorticoid hormone significantly stimulates viral DNA replication. In contrast, addition of EGF results in modest repression of viral DNA replication. These results have important implications for the pathogenesis of HPV infection and suggest that the relative levels of E2, glucocorticoid hormone and EGF may significantly affect the outcome of an HPV infection.

Introduction

Human papillomaviruses (HPVs) are small DNA viruses which infect epithelial cells. Many of them cause benign neoplasias of mucosal epithelia and the DNA of a subgroup of these HPVs is frequently associated with malignant tumours of the cervix. HPV infections can be subdivided into high and low risk infections; in particular, types 16, 18, 31, 33 and 45 are linked to the development of cervical intraepithelial neoplasias (CIN) which may progress to malignancy (zur Hausen & Schneider, 1987). These CIN usually harbour many copies of episomal viral DNA; in contrast, invasive carcinomas often possess mainly integrated HPV DNA sequences (Schwarz et al., 1985; Baker et al., 1987; Matsukura et al., 1989).

In bovine papillomavirus type 1 (BPV-1)-transformed rodent cells, the virus replicates as a stable multicopy plasmid (Law et al., 1981; Gilbert & Cohen, 1987; Ravnan et al., 1992); this has facilitated many of the prototype studies on PV DNA replication. It has been shown that two viral proteins are unequivocally required for supporting the transient replication of plasmids containing the viral origin: the full-length E1 open reading frame protein and the full-length E2 open reading frame protein (Yang et al., 1991; Ustav & Stenlund, 1991; Chiang et al., 1992).

The E1 protein is a nuclear ATP-binding phosphoprotein of about 68 kDa which binds the origin of replication (Sun et al., 1990; Blitz & Laimins, 1991; Wilson & Ludes-Meyers, 1991). In addition, it has been shown that E1 has both ATPase and helicase activities (Seo et al., 1993; Hughes & Romanos, 1993; Bream et al., 1993) and also shares some amino acid homology with SV40 large T antigen.

The E2 protein is a well-characterized major regulator of viral gene expression (Phelps & Howley, 1987; Cripe et al., 1987; Bouvard et al., 1994a). It binds as a dimer to the viral upstream regulatory region (URR) at ACC Ny GGT motifs which are found repeated in the viral enhancer (Androphy et al., 1987). It has recently been demonstrated that at low levels the full-length HPV-16 E2 protein functions as a transactivator of the HPV-16 P97 promoter in cervical keratinocytes, whereas
increased intracellular levels of E2 result in repression of HPV transcription (Bouvard et al., 1994a; Ushikai et al., 1994). Structural studies of the E2 protein have revealed that the E2 transactivator consists of two functional domains linked by a hinged region. The N-terminal region of the protein is approximately 200 amino acids long and includes the transcriptional activation domain; the C-terminal domain is involved in specific DNA binding to the viral URR and also in E2 dimerization (Giri & Yaniv, 1988). Alternative splicing also results in truncated forms of the HPV-16 and BPV-1 E2 proteins which lack the activation domain but encode the DNA-binding domain; these proteins have been shown to function as repressors of viral transcription (Lambert et al., 1987; Bouvard et al., 1994a).

The full-length E2 protein is able to bind to the E1 protein through its N terminus and recent studies have identified two widely separate regions of the E2 protein which are essential for this binding (Storey et al., 1995; Piccini et al., 1995; Hibma et al., 1995). To date, one detailed analysis of regions of the HPV-16 E2 protein important for viral DNA replication has been reported (Sakai et al., 1996). This has facilitated separation of DNA replication and transcriptional activities of the HPV-16 E2 protein. In this report we have performed a further mutational analysis of the HPV-16 E2 protein within regions of the protein not previously analysed. The mutations introduced into E2 consisted largely of proline substitutions which would be expected to perturb the local secondary structure of the predicted α-helices and β-sheet conformation of the protein. These studies have enabled us to dissect further the regions of E2 that are important for transcriptional activity, E1 binding and DNA replication. Contrary to previous suggestions, these studies indicate that the HPV-16 E2 protein consists of a series of functional domains with separable activities, since several mutants have been isolated which retain at least one of the three major activities of the E2 protein. In addition to the regions of E2 important for viral DNA replication, we were also interested in other factors which may regulate viral DNA replication. Therefore, we have performed a series of analyses on the effects of glucocorticoid hormone and EGF on viral DNA replication, since both have been shown previously to modulate viral gene expression (Gloss et al., 1987; Chan et al., 1990; Bouvard et al., 1994b). Our results show that addition of glucocorticoid hormone markedly stimulates the level of viral DNA replication. In contrast, addition of EGF significantly reduces the level of viral DNA replication. Taken together, these results suggest that, in addition to E2, levels of glucocorticoid and EGF within the infected epidermis may significantly affect the outcome of an HPV infection.

Methods

Plasmids. Full-length E2 was cloned into pl/ΔΩ to give pl/ΔΩ 16E2 as described previously (Lees et al., 1990) and consists of nucleotides 2755–3853 from the HPV-16 genome. Mutants of E2 were generated by cloning the full-length gene into M13mp19 and mutagenesis reactions were performed using an in vitro system (Amersham). For single point mutations, 21 base oligonucleotides were used. Where larger deletions were required, 28-mers were used such that a 14 base anchor was present on each side of the sequence which was to be detected. All mutations were confirmed by sequencing.

Protein expression vector pCGE1B, expressing HPV-18 E1 and E2, and expression vector pCGE1BA2 expressing only HPV-18 E1 have been described previously (Remm et al., 1992). The CAT reporter plasmid used in this study, termed 16URR::TK CAT, as well as the plasmid 16URR::CAT containing the HPV-16 origin of replication, have already been described (Bouvard et al., 1994a).

Cells and transfections. 293 cells were cultured in DMEM supplemented with 10% foetal calf serum. Transfection with plasmid DNA was done by standard calcium phosphate precipitation. Transfection efficiency was monitored using a β-galactosidase-expressing plasmid (pCH110, Pharmacia) and typically ranged from 20 to 30%. Dexamethasone (DEX) was used at a concentration of 10−8 M and EGF was used at 50 ng/ml.

CAT assays. Cells were transfected with 5 μg of CAT reporter plasmid and 5 μg of E2 expression plasmid. Cells were harvested after 48 h in 400 μl of 40 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA and subjected to three freeze–thaw cycles, followed by incubation at 65 °C for 10 min. Samples were clarified by centrifugation at 14,000 r.p.m. in a microfuge and protein concentration was estimated by the Bio-Rad protein assay. CAT assays were performed with 1 μg protein incubated with 2.5 μl acetyl-CoA (33 mg/ml) and 1.5 μl [14C]chloramphenicol (50 mCi/mmol, Amersham) in a final volume of 50 μl for 30 min at 37 °C. Following extraction with ethyl acetate, samples were analysed by thin-layer chromatography and visualized by autoradiography. Samples were quantified with a PhosphorImager.

Transient replication assay. Transient replication assays were done with 8 × 105 cells. For the E2 assays 5 μg of either the mutated or wild-type p/ΔΩ 16E2 expression plasmid DNA plus 5 μg of E1 expression plasmid pCGE1BA2 and 1 μg of the replicon DNA 16URR::CAT were transfected into 293 cells. Three days post-transfection, low molecular mass DNA was isolated by the Hirt extraction procedure (Hirt, 1967). Samples were digested overnight with BamHI to linearize the various plasmids and treated with an excess of DpnII to remove the unreplicated, input dam methylated DNA. Total digestion products were separated on a 0.8% agarose gel in Tris–acetate–EDTA buffer. The DNA products were transferred to a Hybond-N membrane and subsequently hybridized to a [32P]-labelled 16URR::CAT probe generated by random priming. Blots were hybridized with the probe overnight at 42 °C in a solution containing 6 × SSC, 5 × Denhardt’s solution, 0.5% SDS, 50% formamide and 500 μg/ml salmon sperm DNA. Blots were washed three times for 30 min at 42 °C in 2 × SSC and 0.1% SDS and then twice for 40 min at 55 °C in a solution containing 0.2 × SSC and 0.1% SDS. Blots were usually exposed for 10 min and quantified on a PhosphorImager.

Western blot analysis. The relative stability of the different E2 mutant proteins was determined by Western blot analysis following transfection of 293 cells. Cells were harvested after 24 h and extracted in a solution of 50 mM HEPES pH 7.0, 250 mM NaCl, 0.1% NP40, 32 μg/ml PMSF and 1% Aprotinin. Protein concentrations were determined using the Bio-Rad protein assay and equal amounts were run on PAGE and transferred to a nitrocellulose membrane. HPV-16 E2 protein was detected using a pool of the anti-E2 monoclonal antibodies TVG 261 and TVG 271 (Hibma et al., 1995), followed by incubation with rabbit anti-mouse biotin conjugate (DAKO) and avidin peroxidase.
plasmid compared with the pJ4, and it is striking how much more E2 is expressed from this. As a positive control for this assay we also included pCMV-E2.

Only M7 appeared to be completely unstable (data not shown). Of the remaining mutants analysed, the expression of the mutant E2 proteins were then determined by Western blot analysis. The results obtained are shown in Fig. 2 and demonstrate that the mutant E2 proteins are relatively stable with respect to the wild-type protein; only M10 appears to be somewhat weaker. Of the remaining mutants analysed, only M7 appeared to be completely unstable (data not shown).

To investigate this we performed a series of transient transfections and DNA replication assays. Human 293 cells were transfected with the different E2 mutants together with the HPV-16URR::TK CAT reporter. After 48 h cells were harvested and CAT activity was measured. Results obtained from a series of assays are shown in Table 1. These results indicate that wild-type protein induced a mean activation of the enhancer of approximately 2.6-fold and this is in agreement with previously published results (Bouvard et al., 1994a). The mutant E2 proteins display a broad range of activities, from close to wild-type (M5 and M11), intermediate (M1) and defective (M4, M7, M9, M8, M10 and M12). This analysis defines regions of the E2 protein around aa 73, 156–159 and 194–197 as being essential for transcriptional activity in the cells chosen for the replication assay, thus allowing a valid comparison between the transcriptional and DNA replication activities of E2 to be made. Human 293 cells were transfected with the different E2 mutants together with the HPV-16URR::TK CAT reporter. After 48 h cells were harvested and CAT activity was measured. Results obtained from a series of assays are shown in Table 1. These results indicate that wild-type protein induced a mean activation of the enhancer of approximately 2.6-fold and this is in agreement with previously published results (Bouvard et al., 1994a). The mutant E2 proteins display a broad range of activities, from close to wild-type (M5 and M11), intermediate (M1) and defective (M4, M7, M9, M8, M10 and M12). This analysis defines regions of the E2 protein around aa 73, 156–159 and 194–197 as being essential for transcriptional activity in human 293 cells.

It is clear from this analysis that a large proportion of the E2 mutants are defective. One possible explanation for this could be the instability of the mutant E2 proteins themselves. To investigate this we performed a series of transient transfections into 293 cells. After 24 h cells were extracted and levels of expression of the mutant E2 proteins were then determined by Western blot analysis. The results obtained are shown in Fig. 2 and demonstrate that the mutant E2 proteins are relatively stable with respect to the wild-type protein; only M10 appears to be somewhat weaker. Of the remaining mutants analysed, only M7 appeared to be completely unstable (data not shown). As a positive control for this assay we also included pCMV-E2 and it is striking how much more E2 is expressed from this plasmid compared with the pJ4Ω-based constructs. This result supports our previous observations which suggested that pCMV-based vectors largely overexpressed the E2 proteins and accounts for the apparent ability to repress transcription, a phenomenon which is not observed when the E2 proteins are expressed from the pJ4Ω vector (Bouvard et al., 1994a).

Identification of the regions of the E2 protein necessary for transient viral DNA replication

In order to assess the activity of the different E2 mutants in the DNA replication assay, 293 cells were co-transfected by

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**Results**

**Transcriptional activation by the HPV-16 E2 mutant proteins**

The structure of the full-length E2 protein can be functionally divided into three different domains: an N-terminal region of the protein which includes the transcriptional activation domain; a central hinge region; and a C-terminal domain involved in specific DNA binding to the viral URR as well as in DNA dimerization. Comparison of the N-terminal domains of the E2 proteins identified highly conserved regions amongst a large number of HPV genotypes; these were chosen for mutational analysis. Fig. 1 shows the location of the mutations which were introduced into the HPV-16 E2 protein.

The E2 mutants were cloned in the pJ4Ω expression plasmid and then assayed for their ability to activate transcription of the E2 responsive reporter plasmid, HPV-16URR::TK CAT (Bouvard et al., 1994a). Although the transcriptional activity of a number of these mutants has been assessed previously, we felt it was necessary to measure their transcriptional activity in the cells chosen for the replication assay, thus allowing a valid comparison between the transcriptional and DNA replication activities of E2 to be made. Human 293 cells were transfected with the different E2 mutants together with the HPV-16URR::TK CAT reporter. After 48 h cells were harvested and CAT activity was measured. Results obtained from a series of assays are shown in Table 1. These results indicate that wild-type protein induced a mean activation of the enhancer of approximately 2.6-fold and this is in agreement with previously published results (Bouvard et al., 1994a). The mutant E2 proteins display a broad range of activities, from close to wild-type (M5 and M11), intermediate (M1) and defective (M4, M7, M9, M8, M10 and M12). This analysis defines regions of the E2 protein around aa 73, 156–159 and 194–197 as being essential for transcriptional activity in human 293 cells.

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<th>Mutant</th>
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* Numbers represent the mean level of transactivation from at least three independent experiments.

Identification of the regions of the E2 protein necessary for transient viral DNA replication

In order to assess the activity of the different E2 mutants in the DNA replication assay, 293 cells were co-transfected by...
standard calcium phosphate precipitation with E1 and E2 expression plasmids, along with an HPV-16 origin-containing plasmid. DNA replication was quantified by purifying low molecular mass DNA 72 h after transfection, cutting it at a unique restriction site in order to convert the products of replication into linear molecules of unit length, and then digesting it with DpnI in order to eliminate all molecules that had not undergone at least one round of replication. Full-length linear plasmid DNA was identified by its migration during gel electrophoresis, followed by Southern blotting and hybridization with pORI. Each experiment included a negative control lane in which cells were transfected only with pORI replicon. Typical results of a DNA replication assay are shown in Fig. 3 and show undigested replicated DNA (indicated by the arrow) and non-replicated input DNA (at the bottom of the gel). The averaged results from multiple assays are shown in Fig. 4. As with the transcriptional assays, the mutant E2 proteins display a variety of phenotypes with respect to their ability to stimulate viral DNA replication. It is clear from this analysis that the integrity of several regions of the E2 protein, as exemplified by the mutants M2 (aa 33), M3 (aa 47), M4 (aa 73), M9 (aa 156–159), M11 (aa 186) and M13 (aa 23–26) are absolutely essential for efficient viral DNA replication. The results with M4 (Pro-73) are in agreement with previous studies and results with the deletion mutants encompassing aa 23–26 and 156–159 are not surprising since they are also defective in their ability to bind E1 (Piccini et al., 1995). However, M11 (aa 186) is particularly interesting since it retains wild-type levels of binding to E1 and close to wild-type levels of transcriptional activity. These results indicate that HPV-16 E2 protein has a role in viral DNA replication other than simply targeting E1 to the origin of replication. Contrary to previous suggestions, even mutations in the E2 protein which are thought to highly perturb the protein conformation, such as an amino acid substitution with a proline or amino acid deletions, are nonetheless suitable for structure–function analysis since many do not lead to the inactivation of all functions. For example, the M12 deletion mutant (aa 194–197) replicates the HPV-16 ori at least as efficiently as the wild-type E2 protein, yet it is defective in its ability to activate transcription. Thus, this mutational analysis has allowed identification of domains essential for both DNA replication and transcriptional activity of E2 in 293 cells, as well as regions which appear necessary only for replication or for transcriptional activity. A summary of the activities of the mutant E2 proteins with respect to E1 binding, transcriptional activity and their ability to support DNA replication are shown in Table 2.

Effects of glucocorticoid hormone and EGF upon viral DNA replication

The URR contains many potential binding sites for cellular transcription factors including Sp1, AP-1, YY1 and NF1 (Gloss & Bernard, 1990; Sibbet & Campo, 1990; Chong et al., 1991; Bauknecht et al., 1992). In addition, the URR contains a consensus glucocorticoid responsive element (GRE) located about 300 bases upstream of the P97 promoter; transcription of the viral early region can be upregulated by glucocorticoids (Gloss et al., 1987). The HPV-16 enhancer is also responsive to the AP-1 c-fos/c-jun dimer (Chan et al., 1990) and EGF has been shown to stimulate AP-1 activity (Cantley et al., 1991). We
were therefore interested in determining whether these regulators of viral gene expression may also regulate viral DNA replication. To do this, cells were transfected as described above with ori-containing plasmid together with E1 and E2 expression plasmids and the effects of glucocorticoid hormone were assessed by the addition of DEX. After 72 h cells were harvested and the level of HPV DNA replication was measured, as described above. The results obtained are shown in Fig. 5. It is clear that addition of glucocorticoid hormone gives rise to an increase of at least 33% in the level of viral DNA replication. The same replication assay was also performed in the presence of EGF and the results obtained are shown in Fig. 6. In this case the results are not as dramatic as those with glucocorticoid but, nonetheless, it is clear that EGF results in a significant decrease of approximately 20% in viral DNA replication. No changes in the proliferation of the 293 cells were detected during the course of these assays. These results suggest that activation of transcription factor binding to the URR can have both positive and negative effects upon viral DNA replication. These results have important implications for regulation of HPV DNA replication in vivo, where levels of EGF and glucocorticoid hormone will clearly influence both the level of viral DNA replication and viral gene expression.

**Discussion**

The HPV E2 proteins are highly conserved throughout different HPV types and have vital roles in both viral DNA replication and controlling viral gene expression. As such, they represent an attractive target for antiviral therapy. In order to understand more fully the structure–function relation-
ships of the E2 proteins with respect to binding of E1, stimulating viral gene expression and enhancing viral DNA replication, we have performed an extensive mutational analysis of the HPV-16 E2 protein. Our results demonstrate that the HPV-16 E2 protein can be considered to consist of a series of modules, with different functional domains of the molecule involved in E1 binding and DNA replication and other regions involved in transcriptional activation. Although there are mutants which demonstrate areas of overlap between these functions it is clear that these activities are separable in a number of mutants.

In the first part of this study we analysed the ability of the different E2 mutants to activate the HPV-16 enhancer. The results from the transcriptional assays are in broad agreement with previously published observations in that large regions of the N-terminal domain of E2 appear to be essential for transcriptional activity (Sakai et al., 1996). Only mutants M5 (Pro-94) and M11 (Pro-186) exhibited levels of transcriptional activity approaching that of the wild-type protein.

We then proceeded to investigate the degree to which the mutant E2 proteins could support transient viral DNA replication. Previous studies have shown that the ability to activate transcription could be separated from the ability to stimulate viral DNA replication (Sakai et al., 1996). Results from the studies presented here support these conclusions and, further, identify additional regions of the E2 protein which are important for viral DNA replication. The importance of the N-terminal region of E2 in both DNA replication and transcriptional activation is highlighted by mutants M2 (Pro-33), M3 (Pro-47), M4 (Pro-73) and M13 (deletion 23–26) which are completely negative for DNA replication and are weak or negative transcriptional activators. Similar mutations to these have been reported previously and although our data largely support those studies (Sakai et al., 1996), some differences do exist. The mutations described here are likely to be more disruptive due to insertion of Pro as opposed to Ala in the study by Sakai et al. In addition, the replication assays described in Sakai et al. require PCR amplification to detect the replicated products; this is not necessary in our study and indicates that the assay described here is more sensitive. Thus, mutant M2 (Pro-33) is in agreement, mutant M3 (Pro-47) is largely defective in our study but the equivalent Arg47Ala mutant exhibits wild-type levels of transcriptional activity and only reduced levels of DNA replication activity. Finally, mutant M4 (Pro-73) is again largely defective in our study but the equivalent Ile73Ala mutant has wild-type DNA replication activity and is defective in the transcriptional assays. The conclusion which can be drawn from these two studies is that this extreme N-terminal region of the transactivation domain of E2 is critical to both DNA replication and transcriptional activation and, depending upon the severity of the mutation introduced, permits only a partial separation of the transcriptional and DNA replication activities of the E2 protein.

We have previously shown that mutant E2 proteins within the region encompassing aa residues 26–47 have reduced E1 binding activity (Piccini et al., 1995) and therefore the reduced ability to support DNA replication is not surprising. However, mutant M4 (Pro-73) has wild-type E1 binding activity and this mutant therefore points to an additional function of E2 other than that of E1 binding in DNA replication. The novel mutants in this analysis have mutations which fall in the latter half of the transactivation domain of the protein. Of these, the most interesting are represented by mutants M11 (Pro-186), M12 (deletion 194–197) and M14 (Ala-100–102). Firstly, the Pro-186 mutation defines an additional region of the E2 protein which is essential for DNA replication, although further mutational analysis will be required to determine the extent of this region. This mutant has wild-type levels of E1-binding activity and almost wild-type levels of transcriptional activity. Therefore, this demonstrates that there are activities of E2 in addition to that of binding E1 which are required for efficient viral DNA replication. In contrast, mutant M14 displays wild-type levels of DNA replication and E1-binding activity, yet has very weak transcriptional activity. Therefore both of the mutations at Ala-100–102 and Pro-186 allow the transcriptional activity of E2 to be separated from its DNA replication activity. This conclusion is further supported by the 194–197 deletion since this mutant displays extremely efficient activity in the replication assay but is defective in the transcriptional assay.

Having dissected the regions of E2 essential for DNA replication we were then interested in investigating the role of additional factors upon viral DNA replication, with particular emphasis on the hormone- and growth factor-responsive elements within the viral URR. Previous studies have shown that both the AP-1 element and GRE present within the HPV-16 enhancer play an important role in regulating the levels of viral gene expression (Chan et al., 1990; Cantley et al., 1991).
Since the ori-containing plasmid used in this study contains the AP-1 sites and the GRE, we assessed the levels of HPV-16 DNA replication in 293 cells following addition of DEX and EGF. Results clearly show that glucocorticoid hormone promotes a significant increase in the level of viral DNA replication. In contrast, EGF has a marked inhibitory effect upon viral DNA replication. These results provide further evidence of the crucial roles of both glucocorticoids and EGF in vivo both with respect to the regulation of viral gene expression and to the regulation of viral DNA replication. Since we cannot rule out whether the effects of glucocorticoid and EGF are due to transcriptional effects on the URR, studies are currently in progress to ascertain whether the effects of the glucocorticoid and EGF are mediated through AP-1 and glucocorticoid receptor (GR) binding to the URR or alternatively by modifications of the cellular DNA replication machinery. This aspect is particularly relevant since previous studies have shown that the GR can modify chromatin structure (Tsai & O’Malley, 1994) and steroid hormones have also been shown to modestly increase the level of SV40 DNA replication (Zuo & Mertz, 1995).

In conclusion, we have defined regions of the E2 protein which are necessary for efficient viral DNA replication. These studies have allowed separation of the transcriptional activity of E2 from that of its role in DNA replication. In addition, we have provided compelling evidence for a role for E2 in viral DNA replication other than that of simply targeting E1 to the origin of replication. Finally, the presence or absence of EGF and glucocorticoid hormones represent alternative mechanisms for modulating the level of viral DNA replication in response to external stimuli.

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