Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids

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ABSTRACT

We have analyzed the chromatin structure of the human $\beta$-globin locus in somatic cell hybrids resulting from the fusion of human non-erythroid cells and mouse erythroleukemia (MEL) cells. In these hybrids, the human adult $\beta$-globin gene, but neither the embryonic nor fetal globin genes, is activated transcriptionally. In addition, the DNase I-resistant $\beta$-like globin locus characteristic of the parental non-erythroid human cells (1,2) is reorganized over an approximately 80 kb region, including the formation of the developmentally stable hypersensitive sites 50 kb 5' and 20 kb 3' of the activated adult $\beta$-globin gene (2,3). These results are consistent with the hypothesis that events occurring at the 5' and/or 3' developmentally stable hypersensitive sites are important, if not necessary, for the activation of the $\beta$-globin locus.

INTRODUCTION

The human $\beta$-like globin gene cluster has been a model genetic locus for the investigation of tissue-specific as well as developmentally regulated gene expression. This locus contains five very similar $\beta$-like globin genes which are transcribed in specific periods of erythroid cell development. All of the $\beta$-like globin genes reside within a 55 kb region on chromosome 11 (reviewed in 4).

Chromatin structure analyses have revealed the presence of tissue specific DNase I hypersensitive sites within the promoters of the $\beta$-like globin genes only during the developmental times when these genes are expressed (1). For example, hypersensitive sites 5' to the $\beta$-globin genes in fetal erythroid cells are not found in adult erythroid cells (1). These changes in chromatin structure, therefore, accompany the developmental phenomena known as switching. In contrast, the entire 55 kb region containing the five globin genes is in a DNase I sensitive chromatin...
conformation in all erythroid cells, regardless of which specific globin gene is expressed (1,2). In addition, erythroid-specific and developmentally stable hypersensitive sites which are not correlated with specific globin gene expression and do not switch have been found 6.1, 10.9, 14.7, and 18 kb 5' to the ε- and 21.8 kb 3' to the β-globin genes (2,32). We hypothesized previously that the formation of these stable sites might be important in the developmental activation of the β-like globin locus (2).

Attempts to understand the mechanisms which regulate the expression of the β-like globin genes have been facilitated by naturally occurring mutant globin alleles which exhibit abnormal patterns of regulation. A specific subset of the (γββ)° thalassemia class of mutations involve deletions of the 5' end of the β-globin cluster that leave all of the remaining globin genes inactive and DNase I-resistant (6). The unexpressed β-globin genes, however, function normally in heterologous transcription assays (7,8). The finding that the DNA juxtaposed into the globin locus as a result of these deletions is DNase I-resistant led to the hypothesis that elements within this region exert a negative effect on the remaining globin genes (6). However, since these deletions remove the region containing the developmentally stable hypersensitive sites 5' to ε, it is also possible that the failure to activate the globin locus is due to the removal of positive elements necessary for this activation early in erythroid differentiation.

In an attempt to understand the relationship between the developmentally stable hypersensitive sites and the expression of the β-globin genes, we have analyzed the β-globin locus chromatin after activation of the adult β-globin gene in somatic cell hybrids. In this system human non-erythroid cells are fused with mouse erythroleukemia (MEL) cells, leading to the activation of the human adult but not the embryonic or fetal globin genes in trans (9). We report here that the trans-activation of the adult β-globin gene is associated with the reorganization of a large region of chromatin, including the formation of the distant developmentally stable hypersensitive sites 5' of ε and 3' to β. These results indicate that activation of the β-like globin genes in these hybrids resembles normal locus activation
and supports the hypothesis that events occurring at these distant developmentally stable hypersensitive sites are important for the activation of this locus.

MATERIALS AND METHODS

Cells used in this study have been described previously (for review see 10). Erythroid enriched fractions from fetal livers and adult bone marrow aspirates were obtained and processed as described (1,2). Hybrids were generated, selected and propagated as described previously (9,11). For the lymphoid cell x MEL cell hybrids, cells harboring human chromosome 11 (which contains the $\beta$-like globin locus) were collected at each passage by panning with a monoclonal antibody raised against a constitutively expressed surface marker encoded on chromosome 11 (9,12). The presence of this surface antigen does not constitute a selective pressure for globin gene expression (12). The M11-X hybrids harbor a t(11;X) translocation that places the X-linked HPRT gene and the $\beta$-globin locus on the same chromosome, allowing for stable maintenance in the presence of HAT media (11,13).

The isolation and DNase I digestion of nuclei (1) and nuclear runoff transcription were also previously described (14). DNase I hypersensitive sites were identified after purifying the DNA from DNase I-treated nuclei, digesting the isolated DNA with restriction enzymes and fractionating the restriction fragments on agarose gels. DNA was transferred to nylon filter membranes as described by Mann and Reed (15). Nicked translated single-copy probes corresponding to one end of a specific restriction fragment (16) were hybridized to filters as described (1) with the addition of 1% SDS and 0.25% powdered milk to the pre- and hybridization buffer.

Cloned DNA probes used in these studies were generously provided by Frank Grosveld (National Institute for Medical Research, UK) Rich Gelinas (Fred Hutchinson) Dixie Mager (Terry Fox, Can.) and P. Powers and Oliver Smithies (U. of Wisconsin). The restriction maps were derived from the published nucleotide sequence of the $\epsilon$-globin gene and its 5' flanking region (17-21).
RESULTS

An alternate chromatin structure in non-erythroid hematopoietic cells.

We previously described a major developmentally stable DNase I hypersensitive site 5' to ε and several minor hypersensitive sites within the same region in erythroid-enriched fractions from fetal livers and adult bone marrows (2). A more detailed analysis using several different cell types indicates that two of these hypersensitive sites are specific for nonerythroid hematopoietic cells. This is demonstrated by comparing the chromatin structure of the 5'ε region in the human erythroleukemia cell line K-562 (22) and the lymphoid cell line Manca (23, see fig. 1). The alternate, non-erythroid, hematopoietic hypersensitive sites in the lymphoid cells are located 5.2 kb and 7.2 kb upstream of the ε-globin gene, approximately 1 kb to either side of the erythroid-specific developmentally stable hypersensitive site located at -6.1. In contrast these hypersensitive sites are not observed in fetal brain (2), fibroblast (fig. 3), or HeLa cells (data not shown) indicating that cells of non-hematopoietic origin are devoid of any type of hypersensitive site within this region. We have observed these non-erythroid sites in B (23) and T (24) lymphoid cell lines as well as in the granulocytic/monocytic cell line HL-60 (25) and in the multipotential, hematopoietic cell line KG-1A (26, data not shown). It is noteworthy that even though the -5.2 and -7.2 non-erythroid hypersensitive sites are present in nonerythroid hematopoietic cells, the region of chromatin containing these sites is DNase I-resistant in all non-erythroid cells (2, see discussion).

Activation of the human β-globin gene in somatic cell hybrids.

Human lymphoid cells (containing the -5.2 and -7.2 hypersensitive sites) and fibroblasts were fused with Friend virus transformed mouse erythroblastoid cells (MEL) (27). In these hybrids the inactive and DNase I-resistant human globin genes are activated in trans by MEL cell factors (9). By in vitro run-off transcription assays (fig. 2), only the adult β-globin gene is activated in these hybrids. These observations confirm previous studies in which the activation of globin genes
FIGURE 1: Alternate chromatin structure in non-erythroid hematopoietic cells. DNA purified from DNase I-treated nuclei was digested with EcoRI, fractionated by electrophoresis in a 1% agarose gel, blotted, and hybridized with a nick translated 1.4 kb EcoRI-Xba fragment. The large (5.4 kb) subband in the Manca lanes results from DNase I cleavage within the probe region. The bands appearing above the 6.5 kb parent band in the K-562 samples are the result of incomplete enzyme digestion. Samples not treated with exogenous DNase I are marked 0 and increasing amount of DNase I is indicated with an arrow. Subbands in "0" lanes result from endogenous nucleases. Each lane contains 15 ug of DNA. The map below indicates the positions of these hypersensitive sites relative to the ε-globin gene. The 6.5 kb EcoRI fragment is represented by a bold line from -2.1 to -8.6.

in similar hybrids was monitored at the steady state RNA and protein levels (9,11).

We next analyzed the chromatin structure of the human adult β-globin gene activated in these hybrids. Nuclei were treated with DNase I and the purified DNA was restricted, electrophoresed and
FIGURE 2: Activation of the human adult β-globin gene in L-MEL hybrids. (Fig. 2A) Alpha 32-P labeled transcription products from isolated nuclei were used to probe a nylon filter containing 5 μg of plasmid DNA containing the large intervening sequences of each of the ε-, γ-, and β-globin genes. pBR322 is used as a negative control, (2A). (2B) Nuclei from the same L-MEL hybrid
used for in vitro nuclear runoff transcription were treated with increasing amounts of DNase I. DNA from these samples was purified and digested with EcoRI. The filter was hybridized with a nick-translated 1.5 kb Bam HI-Nco fragment. The map indicates the position of this hypersensitive site with respect to the \( \delta \)-globin gene.

blotted. EcoRI digested DNA was probed with a 1.5 kb Bam HI-NcoI fragment located immediately 5' to the human \( \delta \)-globin gene. As shown in fig. 2B the hypersensitive site 5' to the \( \delta \)-globin gene which is associated with the expression of this gene in normal human adult erythroid cells (1) is formed in the hybrids. The location of this site has been confirmed by hybridization of the same filter with an IVS 2 probe which indirectly end-labels the same 5.4 kb EcoRI fragment. The presence of this hypersensitive site indicates that a localized rearrangement of previously DNase I-resistant chromatin has occurred. A similar analysis of the \( \varepsilon \) and \( \gamma \)-globin genes has failed to reveal the hypersensitive sites in the promoter regions of these genes (data not shown), consistent with the lack of transcription of these genes in the hybrids, fig. 2A.

Reorganization of the \( \delta \)-globin locus chromatin in globin-expressing hybrids.

It is possible that the reorganization of the \( \delta \)-globin locus in these hybrids is limited to the promoter region of the activated adult globin gene. In order to determine the extent of the reorganization of the \( \delta \)-globin locus in the hybrids, we inspected the chromatin for hypersensitive sites previously found in all normal erythroid tissue.

To identify DNase I hypersensitive sites in the 5' \( \varepsilon \) region, DNA from DNase I-treated nuclei was digested with EcoRI and BamHI, electrophoresed and blotted. A 1.4 kb EcoRI-Xba probe was used to indirectly end-label a 6.5 kb EcoRI fragment at the 5' end as shown in fig. 3A. Normal lymphoid cells contain the -5.2 and -7.2 sites while normal erythroid cells contain the -6.1 site. In the lymphoid cell x MEL cell (L-MEL) hybrids, the erythroid-specific, developmentally stable hypersensitive site at -6.1 is formed, and the non-erythroid sites at -5.2 and -7.2 which are present in the parental lymphoid cells are lost. The fetal liver sample used in this experiment is approximately 50%
FIGURE 3: Reorganization of chromatin in globin-expressing hybrids: 5'ε, -6.1 region. Nuclei from adult and fetal erythroid cell samples, lymphoid cells and lymphoid cell x MEL (L-MEL) hybrids and fibroblast and fibroblast x MEL (M11-X) hybrids were treated with increasing amounts of DNase I. DNA was digested with EcoRI, separated electrophoretically, blotted and probed with 1.4 kb EcoRI-Xba fragment. The erythroid, lymphoid and fibroblast lanes contain 15 ug each. The L-MEL lanes contain 30 ug each. Weak signal in the M11-X lanes was obtained because only 15 ug was used; hybrids are haploid for chromosome 11. The location of these hypersensitive sites is indicated in the map below fig. 1. The adult nucleated red cell preparation is approximately 80% erythroid and the fetal liver preparation is approximately 50% erythroid.
erythroid and 50% other, mostly white, cells. Thus the presence of the nonerythroid hematopoietic sites in this preparation is not surprising. Other fetal liver preparations in which most nonerythroid cells were removed do not demonstrate the nonerythroid sites as prominently (see 2).

We also examined the chromatin structure of the β-like globin locus in hybrids of human non-hematopoietic cells (fibroblasts) and MEL cells. Like the L-MEL hybrids, this hybrid cell line, M11-X, expresses exclusively the adult β-globin genes (11). In contrast to the nonerythroid hematopoietic cells which contain hypersensitive sites flanking the -6.1 erythroid-specific hypersensitive site, the DNase I-resistant region upstream of is devoid of hypersensitive sites in non-hematopoietic cells such as fibroblasts (fig. 3) and brain (2). As shown in fig. 3 the -6.1 kb site found in normal erythroid tissue is reconstituted on the fibroblast-derived chromosome 11 in the MEL environment. For the remainder of the analysis of the β-globin locus chromatin in globin-expressing hybrids, similar results were obtained in both the L-MEL and M11-X cells. Only the L-MEL results are presented in the figures below.

The adjacent upstream region, represented on a 6.5 kb restriction fragment, is bordered by an EcoRI site at -9.1 and a BamHI site at -15.6. Probes were used that indirectly end-labeled either the 5' or 3' end of this fragment and the results are shown in fig. 4. Using a 1.3 kb BamHI-Hind III probe a 4.7 kb and a 5.7 kb subband are seen in the fetal and adult erythroid tissue. Interestingly, the 5.7 kb subband is absent in the erythroleukemia cell line K-562, and no DNase I hypersensitive sites are found in this interval in the lymphoid cells. This difference demonstrates the importance of examining normal erythroid tissue as well as cell lines. To unambiguously assign positions for these hypersensitive sites the same filter was reprobed with either a 1.9 kb Hind III, a 0.8 kb Pvu II-EcoRI or a 0.3 kb Nco-Hind III fragment, each of which end-labels the 3' end and produces a pattern of subbands identical to that seen in fig. 4B. In the erythroid tissue subbands of 1.8 kb and 5.7 kb were found. The K-562 cells do not contain the 5.7 kb subband, but do show the 1.8 kb subband as well as a subband at 2.5 kb which is
FIGURE 4: Reorganization of chromatin in hybrids: 5' e, -10.9 and -14.7 region. In fig. 4A, the erythroid cell line K-562 is compared to normal erythroid tissue and to lymphoid cells. DNA from DNase I-treated nuclei was digested with EcoRI and BamHI, fractionated, blotted and probed with a nick-translated 1.3 kb BamHI-HindIII fragment. In fig. 4B a similar experiment which includes material from the L-MEL hybrids was probed with a 1.9 kb HindIII fragment from the opposite end of the same 6.5 kb BamHI- EcoRI restriction fragment. The location of the hypersensitive sites in this interval are shown in 4C. Map coordinates are from the e-globin transcription initiation site. Downward arrows above the line represent hypersensitive sites from 4A. Upward arrows below the line represent hypersensitive sites from 4B. The (K) below the arrow in 4C indicates a site found predominantly in K-562 cells.
greatly reduced or absent in the erythroid tissue. Thus the K-562 cells differ from normal erythroid cells within this region by the absence of the -14.7 site and the presence of a minor site at -11.6. In addition to being less hypersensitive to DNase I than the nearby -10.9 site, the -11.6 site is unusual in that it is more easily seen with probes at or near the EcoRI end of this restriction fragment. Although several possible explanations exist for the unusual features of the K-562-specific -11.6 site, we do not understand these phenomena and are currently attempting to determine the significance of these observations. In the globin-expressing L-MEL hybrids the subbands found indicate that this region of chromatin is reorganized in the hybrids to resemble that of normal erythroid tissue, rather than that of the erythroleukemia cell line K-562.

We continued our investigation of the chromatin structure 5' to the ε-globin gene by analyzing the hypersensitive sites within a 3.4 kb Bam HI fragment whose endpoints were 15.8 and 19.2 kb upstream of ε (fig. 5). This fragment was indirectly end-labeled at the 3' end with a 1.6 kb Xba-Bam HI probe. When this probe is hybridized to BamHI digested DNA purified from DNase I-treated nuclei, a 2.2 kb subband is seen in the erythroid tissue, as well as in the globin-expressing L-MEL hybrids. This tissue specific, developmentally stable hypersensitive site is located 18 kb 5' to the ε-globin gene.

Next we investigated the chromatin structure of the region containing the developmentally stable hypersensitive site downstream of the β-globin gene to determine if the 3' end of the β-globin locus was reorganized, as well, in the globin expressing hybrids. Purified DNA from DNase I-treated nuclei was digested with Pst and Hind III. A 6.2 kb Pst-Hind III fragment located 16.5 kb to 22.7 kb 3' to the β-globin gene was indirectly end-labeled with a 3.0 kb Pst-EcoRI probe. The results shown in fig. 6 identify a prominent subband of 5.3 kb and a minor one of 4.2 kb can be seen faintly in fetal samples. These correspond to hypersensitive sites 21.8 kb and 20.7 kb 3' to β (when measuring from the β-globin transcription initiation site). Although the sensitivity of these sites is reduced in the hybrids, we do detect the 5.3 kb subband.
FIGURE 5: Reorganization of chromatin in hybrids: 5'$\epsilon$, -18 region. Analysis of erythroid, lymphoid and hybrid, L-MEL, chromatin. BamHI digested DNA is probed with a 1.6 kb Xba-BamHI fragment. The map coordinates are from the $\epsilon$-globin transcription initiation site.

DISCUSSION

Previous work has identified erythroid-specific, developmentally stable hypersensitive sites in fetal and adult erythroid tissue whose roles in the events leading to the expression of the $\beta$-globin genes are unclear (2,3). The fact that these sites are erythroid-specific yet not associated with the transcription of specific globin genes led us to wonder what possible relationship exists between these distant regions and the regulation of the $\beta$-globin genes. As a first approach to understanding the relationship between the developmentally stable sites and globin gene expression, we sought to determine whether trans-activation of the adult globin gene following chromosome-mediated transfer of the $\beta$-globin locus into MEL cells involved a localized reorganization of the chromatin adjacent to the
FIGURE 6: Reorganization of chromatin 3' to β. Adult and fetal erythroid tissue, lymphoid, and lymphoid x MEL hybrid cells were analyzed for hypersensitive sites in a 6.2 kb Pst-HindIII fragment. DNA was digested with HindIII and Pst, fractionated, blotted and hybridized with a 3.0 kb Pst-EcoRI probe. The 6.2 kb parent fragment is shown as a bold line in the map below the fig. and extends from +16.5 to +22.7. The coordinates of the map are distance from the β-globin transcription initiation site.

activated adult globin gene, or whether the entire repertoire of globin-locus hypersensitive sites are formed.

In normal erythroid cells, regardless of the developmental stage, DNase I-sensitive chromatin extends at least 50 kb 5' to the adult β-globin gene (2, and unpublished results). It has been postulated that the distant hypersensitive sites 5' to ε and 3' to β constitute boundaries of erythroid-specific, DNase I-sensitive chromatin (3). We have determined that when the β-globin genes are trans-activated in somatic cell hybrids, all of the tissue-specific, developmentally stable hypersensitive sites found in normal erythroid cells, both 5' and 3' to the locus, are reformed (summarized in fig. 7). Therefore, trans-activation of the β-globin genes from nonerythroid cells by the MEL environment
produces not only a pattern of $\beta$-globin gene expression that resembles normal adult globin gene expression, but also leads to the formation of distant chromatin structures that are characteristic of normal human erythroid cells. The fact that these very distant hypersensitive sites are found in the (adult) globin-expressing hybrids supports the hypothesis that expression of any of the $\beta$-like globin genes may require a restructuring of the chromatin that encompasses the entire $\beta$-globin locus, perhaps initiated by events occurring at the 5' and 3' hypersensitive sites. We would term these sites Locus Activation Regions (LARs) to describe the role they are postulated to play in the regulation of $\beta$-globin gene expression.

This Locus Activation model implies that removal of the sites 5' to $\epsilon$ and 3' to $\beta$ would disrupt or prevent the expression of any of the genes within the $\beta$-globin locus. Consistent with this hypothesis is the observation that deletions removing the region containing the 5' developmentally stable hypersensitive
sites result in the failure to activate the remaining, intact β-globin genes (i.e. English (5) and Dutch (6) (γ6β)° thalassemias, references in 28). Interestingly, deletion of the distant 3' β hypersensitive site which occurs in deletional forms of Hereditary Persistence of Fetal Hemoglobin (HPFH) does not prevent the developmental activation of the β-globin locus (29). However, in the deletional HPFHs, an erythroid-specific developmentally stable hypersensitive site, which is normally over 100 kb 3' to the β-globin gene, is introduced into the β-globin locus as a result of the deletion (Elder and Groudine, unpublished results). Thus, either the 3' site is not required for the developmental activation of the β-globin locus or its function is replaced by sequences juxtaposed as a result of the deletion (37).

Previous experiments using functional assays to identify the 5', internal, and 3' sequences important in the regulation of the adult β-globin gene did not include the regions identified as developmentally stable hypersensitive sites (30-32,34). Even stable transformants containing cosmid size DNAs that included all of the structural genes within the β-globin locus stopped short of the 5' c, -6.1 hypersensitive site (30). If the establishment of a specific chromatin domain is required for high level expression of the β-globin genes, attempts at reproducing normal regulation will depend on the formation of this domain. This may, in part, account for the difficulties encountered by investigators attempting to reproduce normal as well as mutant globin gene expression. For example, tissue and stage specific expression of the human β-globin gene can be achieved in transgenic mice by inclusion of internal and proximal regulatory sequences previously identified via short and long term transfection studies. Yet the levels of expression of the human transgenes are much lower than the endogenous mouse β-globin genes (26). In addition, variations in the levels of expression of the human β-globin transgene have been attributed to position effects and suggest that these injected DNAs may lack information necessary to establish an efficient erythroid-specific chromatin domain. Indeed, preliminary transgenic experiments reveal that a human β-globin transgene is expressed at high levels in a
position independent fashion when the developmentally stable
hypersensitive sites are included (38). Thus it appears that
normal regulation of the globin genes involves cis-acting, gene-
proximal regulatory elements for appropriate tissue-type and
developmental specificity as well as a chromatin configuration
that contributes by an unknown mechanism to the utilization of
these elements. The evidence compiled from the deletional
thalassemias, transfection and transgenic experiments, and our
analysis of the chromatin in globin-expressing hybrids suggest
that the 5' hypersensitive sites may be involved in establishing
the erythroid-specific chromatin structure of the β-globin locus.

The use of transgenic mice as well as mouse cell lines to
study human globin-gene expression is based on the assumption
that the regulatory signals for these genes in mouse and man are
equivalent. The appropriate temporal and spatial patterns of
human globin gene expression in transgenic mice and mouse
erythroid cells support this. Our data demonstrate that the β-
globin chromatin domain reformed in hybrid-activated non-
erthroid cells is identical to the chromatin domain found in
normal human erythroid cells. This indicates that the signals
and events regulating the β-globin locus and the β-globin genes
are similar between mouse and man. From this we extrapolate that
the mouse β-globin genes are regulated by a locus activation
region homologous in function, if not in structure, to the human
β-globin LARs described in this report.

Finally, we have identified hypersensitive sites in non-
erthroid, hematopoietic cells but not in other non-hematopoietic
cells such as brain or fibroblasts. These two sites bracket the
−6.1 kb hypersensitive site (fig. 1), a potentially interesting
alternative structure perhaps formed during non-erythroid
hematopoiesis. The erythroid-specific, developmentally stable
−6.1 kb hypersensitive site (fig. 1), a potentially interesting
alternative structure perhaps formed during non-erythroid
hematopoiesis. The erythroid-specific, developmentally stable
hypersensitive sites are associated with preferential DNase I-
sensitivity of the β-like globin locus chromatin, whereas the
non-erythroid hematopoietic sites flanking the −6.1 site are
found in cell types in which this region is DNase I-resistant.
Thus, it appears that three mutually exclusive chromatin structures can form in the region extending 5' from ε. One, present in erythroid cells in which the chromatin is DNase I-sensitive, contains the 5'ε and 3'β developmentally stable hypersensitive sites. The second, found in hematopoietic cells other than erythroid, is represented by two non-erythroid hypersensitive sites 5' to ε within DNase I-resistant chromatin. It is an intriguing possibility that the presence of these sites in non-erythroid hematopoietic cells is somehow associated with the suppression of an active β-globin chromatin structure in cells descended from a common pluripotent stem cell. The third, represented by the absence of any nuclease hypersensitive sites, as seen in brain, fibroblasts and HeLa cells may reflect the completely repressed nature of this region throughout the lineage history of non-hematopoietic tissue. Further elucidation of the properties of these sequences, principally whether or not they play a regulatory role in the establishment of transcriptionally active chromatin in erythroid cells, will be important in understanding the activation of the β-globin locus during development.

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