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In Nondividing Cells, Histone $H1^0$ Is Synthesized and Deposited onto Chromatin without Accompanying Phosphorylation†

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ABSTRACT: The phosphorylation of H1 histone subfractions was measured in mouse neuroblastoma cells stopped from dividing by three treatments that block cell division: 5 mM butyrate, 2% dimethyl sulfoxide, and serum withdrawal. H1 histone phosphorylation decreased in response to all three treatments, but the response differed in its timing and its extent for the different H1 subfractions. The different decreases in phosphorylation correlated well with the differential decreases in biosynthesis of the individual H1 subfractions; however, an exception to this parallel decrease in synthesis and phosphorylation was observed in the case of histone $H1^0$. Phosphorylation of $H1^0$ was absent in each of the three treatments after 2 days, despite the continued synthesis and deposit of $H1^0$ on the chromatin. Thus, despite the fact that $H1^0$ was being synthesized and that the other newly synthesized H1 subfractions were phosphorylated at this time, the phosphorylation of $H1^0$ became uncoupled from its synthesis after prolonged treatments blocking cell division.

Phosphorylation of H1 histone was first observed in 1966 (Kleinsmith et al., 1966; Ord & Stocken, 1966) and is known to occur at multiple sites on the H1 molecule (Langan, 1982). Although there is some minor hormonally dependent phosphorylation that is independent of the cell cycle (Langan, 1971), almost all histone H1 phosphorylation is correlated with the cell cycle. Major H1 histone phosphorylation has been observed during S phase when DNA replication and most H1 synthesis occurs and during late G_2, immediately prior to the beginning of metaphase (Bradbury et al., 1974). In dividing cells the G_2 phosphorylation accounts for the majority of phosphorylation on H1 molecules, but S-phase phosphorylation is a subtantial fraction. A modest amount of phosphorylation also occurs late in G1 (Gurley et al., 1974). Although the exact role of phosphorylation is not understood, some suggestions have been put forward that distinguish between possible roles of G_2- and S-phase phosphorylation.

The notion that phosphorylation of H1 histone in late G_2 phase of the cell cycle is related to chromosome condensation and the initiation of mitosis has been proposed because of coincidence of the two events (Gurley et al., 1974) and because of the advance of mitosis by exogenous kinase (Bradbury et al, 1974). Moreover, a temperature-sensitive mutant hamster cell line characterized by premature chromosome condensation was found to exhibit extensive phosphorylation of both histones H1 and H3 at the nonpermissive temperature when the premature chromosome condensation occurred (Inglis et al., 1976).

In addition to its occurrence in the late G_2 phase of the cell cycle, phosphorylation of H1 has been observed in S phase, coincident with histone synthesis (Balhorn et al., 1972; Gurley et al., 1974). In this regard, Sung et al (1977) observed that deposition of histone H5 onto the chromatin of chicken erythrocytes is linked to its prior phosphorylation (Sung et al., 1977; Wagner et al., 1977). These workers suggested that phosphorylation may modulate the interactions of histone and DNA while newly synthesized chromatin is annealed to its nature structure [see also Louie & Dixon (1972) and Ruiz-

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Carrillo et al. (1975)]. Although the overall level of phosphorylation is much higher in G_2 (Gurley et al., 1975), the fact that the same sites seem to be phosphorylated in G_2 as in S makes fundamental differences in the roles of phosphorylation seem less likely.

We have been investigating the synthesis and turnover of H1 histones in mouse neuroblastoma cells undergoing treatments that block cell division, essentially eliminating both S phase and G_2. The H1 histones of this cell line can be resolved easily into three subfractions, whose synthesis continues in the absence of cell division, albeit at lower levels, and this system provided an opportunity to test the linkage between H1 synthesis and phosphorylation in the absence of both S phase and G2 and to do so for the H1 subfractions individually.

Materials and Methods

Cell Culture. Mouse neuroblastoma cells NIE-115 (from Marc Kirschner, University of California, San Francisco) were grown on 100 × 20 mm tissue culture dishes at 37 °C and 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM) and 10% fetal calf serum. For the inhibitor studies, this medium was further supplemented with 500 μg/mL sodium butyrate (5 mM) or 1 mL of spectrograde Me_2SO[1]/50 mL of culture medium (250 mM). Medium was changed every day for the first 4 days of culture and every second day thereafter.

Labeling of Histones. For the metabolic studies involved in this paper, the medium was removed from the plate, and the cells were washed once with phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4). The cells were then incubated at 37 °C for the pulse period in 3 mL of either lysine-free or phosphate-free medium supplemented with either 10 μCi of L-[^{14}C]lysine (ICN) or 0.6 mCi of [^{32}P]orthophosphate (Amersham), plus 10% fetal calf serum, 2 mM glutamine, and an inhibitor when appropriate.

Histone Extraction. The outer cell membranes were lysed on each plate of cells by a 3-min incubation at room temperature in 10 mL of buffer (0.25 M sucrose, 50 mM Tris, 25 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.5) plus 0.1% Triton X-100. The nuclei were pelleted by a 5-min spin at 1000*g* and then resuspended in 1 mL of 0.4 N H_2SO_4 (for total histones) or 0.25 mL of 5% perchloric acid (for H1 histones). Following an overnight incubation at 4 °C, cell debris was pelleted by a 10-min centrifugation at 12000*g*, and proteins in the supernatant were precipitated at 20% trichloroacetic acid. The precipitate was washed with ethanol and air-dried.

Gel Electrophoresis and Analysis. Electrophoresis was done in SDS–polyacrylamide gels according to Laemmli (1970), with either a 12.5% or a 15% acrylamide separating gel. The gels were stained with Coomassie blue and destained as previously described (Pehrson & Cole, 1982) and scanned on a Kratos SD3000 xenon lamp spectrodensitometer with an attached Hewlett-Packard 3380A integrator. Autoradiograms were made by drying these gels and exposing them to Kodak X-Omat XAR-5 film. To measure specific activities, the gel bands were excised from the gel, dissolved in 1 mL of 30% H_2O_2 by heating at 75 °C for 4 h, mixed with 10 mL of Aquasol scintillation fluid (Amersham), and then counted. Specific activities were then calculated from the radioactivity (cpm) of the gel band divided by its stain value from the densitometer.

Measurement of DNA Synthesis. DNA synthesis was measured by incubating cells for 3 h in cell culture media containing 0.5 mCi/mL [^{3}H]thymidine (2 Ci/mmol, New England Nuclear). The DNA content of the cells was measured by the diphenylamine assay as described by Adams (Adams, 1980). An aliquot of the material used for the DNA assay was suspended in Aquasol scintillation fluid and counted, and the specific activity of DNA was calculated from the cpm incorporated per microgram of DNA.

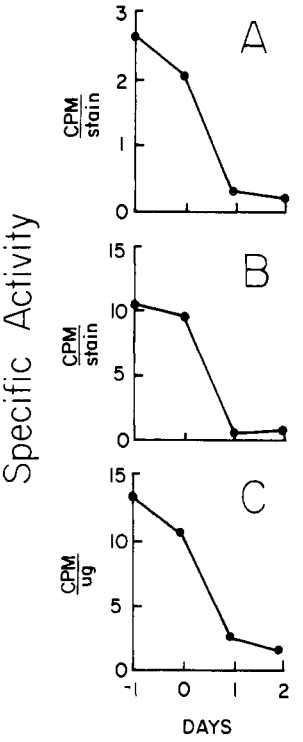

FIGURE 1: Comparison of rates of histone phosphorylation and DNA synthesis after sodium butyrate treatment. Incorporation of inorganic [^{32}P]phosphate into histone proteins or of [^{3}H]thymidine into DNA during the first 48 h of butyrate treatment. Incorporation of both isotopes was measured after a 3-h pulse as described under Materials and Methods. Treatment with 5 mM butyrate was begun on day 0; day −1 represents rapidly growing cells sampled 24 h before the treatment was begun. The 3-h pulse of isotope for day 0 was started when butyrate treatment began. (A) ^{32}P incorporation into H1 histones; (B) ^{32}P incorporation into core histones; (C) [^{3}H]thymidine incorporation into DNA.

Results

Histone Phosphorylation Decreases in Cells Treated with Sodium Butyrate. Cell division in mouse neuroblastoma cells was first inhibited by addition of 5 mM sodium butyrate to the culture media. Phosphorylation was measured in the butyrate-treated cells by incorporation of [^{32}P]orthophosphate into the histone proteins. At each time point during the first 48 h of butyrate treatment, an aliquot of cells was given growth medium that contained radioactive phosphate (200 μCi/mL) as the only source of phosphate and then grown for an additional 3 h. The cells were harvested at the end of the 3-h pulse; the histones were extracted with 0.4 N H_2SO_4 and analyzed on SDS–polyacrylamide gels for specific activity of the histones. DNA synthesis during this period was also measured by incorporation of [^{3}H]thymidine (500 μCi/mL).

As seen in Figure 1, butyrate treatment caused a decrease in the rate of ^{32}P incorporated into both the H1 histones (panel A) and the core histones (panel B) concomitant with the inhibition of DNA synthesis (panel C). After 48 h in butyrate, the rate of phosphorylation had dropped 93% for H1 and 86% for the core histone relative to the rate in rapidly growing cells. This is in keeping with the observations of Boffa et al. (1981)

[1] Abbreviations: Me_2SO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

Table I: Comparison of the Decrease in ^{32}P and [^{14}C]Lysine Incorporation into the H1 Histone Subfractions after 24 h of Butyrate Treatment[a]

H1 subfraction	time (h)	^{32}P incorporation			[^{14}C]lysine incorporation		
		cpm	sp act.	relative decrease	cpm	sp act.	relative decrease
H1ab	0	4841	17.0		2376	2.3	
	24	492	1.5	11.4	687	0.3	7.7
H1c	0	1120	22.4		613	1.8	
	24	280	4.1	5.5	841	0.5	3.5
$H1^0$	0	195	5.3		353	2.5	
	24	48	1.1	4.9	438	0.6	4.2

[a] Values were calculated from measurements of the incorporation of inorganic [^{32}P]phosphate and [^{14}C]lysine into the H1 histone subfractions during 3-h pulses given to populations of rapidly growing cells and cells treated for 24 h with 5 mM butyrate. The specific activity of the H1 histone subfractions in the rapidly growing and 24-h butyrate-treated cells was calculated for each isotope as described in the text.

that H1 phosphorylation in HeLa cells decreased 98% and core histone phosphorylation decreased 90% after 15 h in 5 mM butyrate. Similarly, D'Anna et al. (1980) reported a 89% decrease in H1 phosphorylation in CHO cells after 24 h in 5 mM butyrate-containing media. The decrease in all three curves of Figure 1, between days −1 and 0, was due to the fact that the cell cultures were approaching confluence, and so their rate of cell division was decreasing.

The phosphorylation of the individual H1 subfractions was monitored during the first 24 h of butyrate treatment. Although the H1 histone of NIE-115 mouse neuroblastoma cells can be resolved into four subfractions by two-dimensional acetic acid/SDS gel electrophoresis, in the one-dimensional SDS gel analysis used in this work, the first two H1 subfractions, H1a and H1b, were not separated and were analyzed together. Since previous work in this laboratory showed that H1a and H1b behave similarly to one another metabolically (Pehrson & Cole, 1982), combining the two H1 subtypes in analysis is not likely to give misleading results. The decrease in phosphorylation of the individual H1 subfractions during the first 24 h of butyrate treatment is shown in Figure 2. The phosphorylation rates of the H1 subfractions decreased from different initial levels to different final levels, and at different rates.

As the rates of phosphorylation did not reflect simply the absolute amounts of the individual H1 subfractions on the chromatin, the changes in rates of phosphorylation of the individual subfractions were compared with changes in their rates of biosynthesis as butyrate treatment progressed. Cells were incubated for 3 h in media containing [^{14}C]lysine (0.3 μCi/mL) both before and after 24 h of butyrate treatment. The cells were harvested at the end of the 3-h pulse, and the histones extracted with 0.4 N H_2SO_4 were analyzed on SDS–polyacrylamide gels. As seen in Table I, the extent of decrease in phosphorylation of the individual H1 subfractions roughly paralleled the extent of decrease in their biosynethesis. Thus, as in the case of phosphorylation, the rate of biosynthesis of H1ab decreased twice as much as that for H1c and $H1^0$ in the initial 24 h of butyrate treatment. These results indicate a general trend among the first 24 h of butyrate treatment, the components of which have been observed by others: (1) butyrate blocks cell division in G_1, inhibiting DNA synthesis (D'Anna et al., 1980); (2) H1 synthesis is also reduced, since most histone biosynthesis is linked to DNA synthesis (Delegeane & Lee, 1982); (3) phosphorylation of the H1 subfractions is reduced since phosphorylation occurs in S phase and late G_2, which these cells no longer traverse.

Synthesis of $H1^0$ without Concomitant Phosphorylation. NIE-115 cells may be maintained in butyrate-containing media for periods as long as 3 weeks and remain viable. After 24 h of butyrate treatment, the synthesis of the H1 histones still occurs, although at a much lower level than in rapidly growing cells (Pehrson & Cole, 1982). Surprisingly, as seen in Figure 3, the phosphorylation of $H1^0$ was undetectable on the second day of treatment, and thereafter. A more exact measurement of the phosphorylation and synthesis of the H1 subfractions

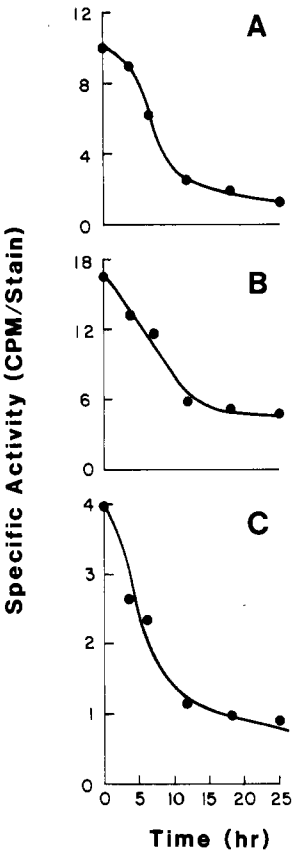

FIGURE 2: Decreases in phosphorylation of the individual H1 subfractions after treatment with 5 mM sodium butyrate. Cells were labeled for 3 h in media containing inorganic [^{32}P]phosphate at various times during the first 24 h of butyrate treatment. The cells were harvested at the end of the 3-h pulse, the nuclei were isolated, and the histones were extracted with 0.4 N H_2SO_4. Histones were electrophoresed on SDS–polyacrylamide gels, which were then stained and scanned to measure protein. The individual H1 subfraction bands wer then excised and dissolved in 30% H_2O_2, and radioactivity was measured. (A) H1ab; (B) H1c; (C) $H1^0$.

Table II: Comparison of Incorporation of [^{14}C]Lysine and ^{32}P into H1 Histone Subfractions after 4 Days of Butyrate Treatment[a]

H1 subfraction	[^{14}C]lysine (sp act.)	^{32}P (sp act.)
H1ab	1.3	1.1
H1c	1.8	1.2
$H1^0$	1.4	≤0.05

[a] Values were calculated from measurements of the incorporation of inorganic [^{32}P]phosphate and [^{14}C]lysine into the H1 histone subfractions during a 3-h pulse given to cells treated for 4 days with 5 mM butyrate. The value for ^{32}P incorporation into $H1^0$ is at background levels for this assay.

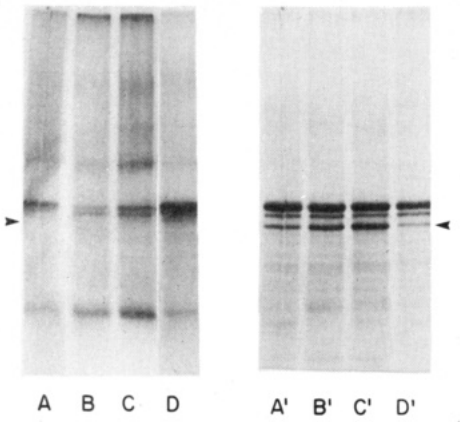

FIGURE 3: Phosphorylation of H1 histones after prolonged butyrate treatment. Cells were labeled for 3 h with inorganic [^{32}P]phosphate on various days of butyrate treatment. The histones were isolated by extraction with 5% perchloric acid and electrophoresed on an SDS–polyacrylamide gel, which was then autoradiographed. The position of H1^0 is marked by an arrowhead. (Left panel) autoradiogram; (right panel) Coomassie blue stained SDS gel; (lanes A and A′) 2 days in butyrate; (lanes B and B′) 4 days in butyrate; (lanes C and C′) 8 days in butyrate; (lanes D and D′) rapidly growing cells.

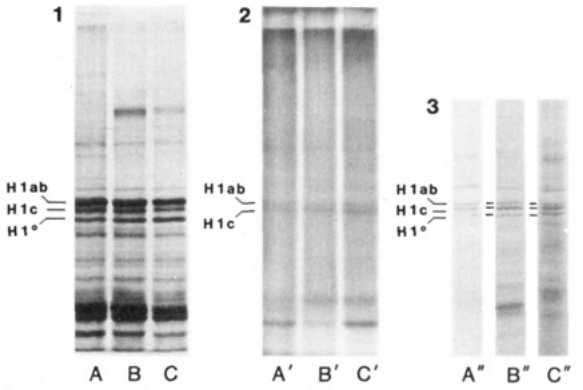

FIGURE 4: Synthesis and phosphorylation of H1 histone subfractions in cells having undergone 4 days of various treatments that block cell division. Cells were stopped from dividing for 4 days by treatment with either 5 mM butyrate or 2% Me_2SO or withdrawal of serum. At the end of these 4-day treatments, cells were labeled with either inorganic [^{32}P]phosphate or [^{14}C]lysine for 3 h. The histones were then isolated by extraction with 0.4 N H_2SO_4 and elelectrophoresed on SDS–polyacrylamide gels, which were then autoradiographed. (Panel 1) Coomassie blue stained SDS gel of ^{32}P-labeled histones from 4-day cells; (panel 2) autoradiogram of panel 1; (panel 3) autoradiogram of [^{14}C]lysine-labeled histone proteins from 4-day cells. In each panel, the three treatments are (A) no serum, (B) Me_2SO, and (C) butyrate.

after prolonged butyrate treatment is given in Table II. In this experiment, cells were grown for 4 days in butyrate and then labeled for 3 h by addition to the culture media of either [^{32}P]orthophosphate (200 μCi/mL) or [^{14}C]lysine (3.3 μCi/mL). The histones isolated by 0.4 N H_2SO_4 extraction were analyzed on SDS–polyacrylamide gels. Both H1ab and H1c were phosphorylated at rates comparable to their biosynthetic rates during this 8-day period (Table II); however, H1^0 was not phosphorylated at all after 2 days in butyrate, despite the fact that it was being synthesized at approximately the same rate as the other H1's (Table II). Thus, prolonged treatment of these cells with butyrate uncoupled the synthesis and phosphorylation of H1^0 while the other newly synthesized H1's continue to be phosphorylated.

It might be asked if this decoupling of H1^0 synthesis and phosphorylation is unique to butyrate treatment, which has pleiotropic effects on cells, including changes in histone acetylation, induction of specific enzymes, and reversion of the transformed characteristics of many cell lines (Kruh, 1982; Prosad & Sinha, 1976). Therefore, we tested two other methods of blocking cell division—treatment of the cells with culture media containing 2% Me_2SO and withdrawal of serum from the culture media. As shown in Figure 4, 4 days of either treatment gave results comparable to those obtained after 4 days of butyrate treatment, with phosphorylation of H1ab and H1c continuing to occur without detectable phosphorylation of H1^0, even though the latter was synthesized in all three treatments at approximately the same levels as the other H1's (Figure 4, panel C). Therefore, the uncoupling of the synthesis of H1^0 from its phosphorylation is not an artifact of butyrate treatment but is a characteristic of basal histone metabolism in quiescent cells in culture.

Discussion

Since phosphorylation of H1 histone is known to occur along with histone synthesis in cells traversing the cell cycle, it was not surprising to find a similar correlation in cells prevented from cycling by butyrate and other treatments. Although the individual H1 subfractions decreased their phosphorylation rates to different levels after cell division was blocked, with one exception the decreases matched decreases in H1 synthesis rates so that the correlation known for rapidly dividing cells applied to nondividing cells as well.

The exception referred to above was histone H1^0, which is best known for its increased levels in nondividing cells (Panyim & Chalkley, 1969). Like histones H1ab and H1c, histone H1^0 continued its synthesis (at a lower rate) after cell division was blocked, but unlike H1ab and H1c, its phosphorylation ceased. Although phosphorylation in the absence of synthesis has been reported previously (Johnson & Allfrey, 1978; Billings et al., 1979; Glover et al., 1981; Langan, 1968), to our knowledge this is the first report of H1 synthesis without accompanying phosphorylation.

To some extent the mechanism, and perhaps the role, of histone H1^0 phosphorylation seems different from those of the other H1 subfractions. A difference in mechanism might represent simply independent kinases, or it might reflect differences between H1^0 and the other H1's in the way they are transported and deposited on the chromatin. An attractive notion of long standing (Sung et al., 1977) is that the deposit of H1 histone onto chromatin occurs while the histone is in the phosphorylated state. The partial masking of the cationic surface of H1 by phosphoryl groups would allow an annealing of the protein into its proper position, and then, dephosphorylation would stabilize the complex. While such a mechanism might still be entertained for the other H1's in dividing and nondividing cells, it obviously cannot apply to the deposit of H1^0 onto chromatin in nondividing cells.

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Chromatin Structure of the Chicken Lysozyme Gene Domain As Determined by Chromatin Fractionation and Micrococcal Nuclease Digestion†

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ABSTRACT: The chromatin structure encompassing the lysozyme gene domain in hen oviduct nuclei was studied by measuring the partitioning of coding and flanking sequences during chromatin fractionation and by analyzing the nucleosome repeat in response to micrococcal nuclease digestion. Following micrococcal nuclease digestion, nuclei were sedimented to obtain a chromatin fraction released during digestion (S1) and then lysed in tris(hydroxymethyl)aminomethane–(ethylenedinitrilo)tetraacetic acid–[ethylenebis(oxyethylenenitrilo)]tetraacetic acid and centrifuged again to yield a second solubilized chromatin fraction (S2) and a pelleted fraction (P2). By dot-blot hybridization with 14 specific probes, it is found that the fractionation procedure defines three classes of sequences within the lysozyme gene domain. The coding sequences, which partition with fraction P2, are flanked by class I flanking sequences, which partition with fractions S1 and P2 and which extend over 11 kilobases (kb) on the 5′ side and probably over about 4 kb on the 3′ side. The partitioning of class II flanking sequences, which are located distal of class I flanking sequences, is different from that of class I flanking sequences. Coding sequences lack a canonical nucleosome repeat, class I flanking sequences possess a disturbed nucleosome repeat, and class II flanking sequences generate an extended nucleosomal ladder. Coding and class I flanking sequences are more readily digested by micrococcal nuclease than class II flanking sequences and the inactive β^A-globin gene. In hen liver, where the lysozyme gene is inactive, coding and class I flanking sequences fractionate into fractions S2 and P2. Chromatin fractionation of steroid-induced and deinduced chick oviduct nuclei shows a close correlation between active transcription of the lysozyme gene and enrichment of coding sequences in fraction P2. Our results indicate that the partitioning of coding lysozyme gene sequences with low-salt insoluble nuclear material relates to the transcriptional process along these sequences.

The packaging of transcribed genes into a less condensed chromatin structure has been visualized by electron microscopy (Beermann, 1952; Foe et al., 1976) and has been investigated by use of nuclease digestion (Weintraub & Groudine, 1976). Active genes and genes that have been transcribed exhibit an elevated sensitivity to digestion with various nucleases. A more detailed analysis performed on many genes revealed that often a distinct length of flanking sequences is equally as sensitive to nuclease digestion as the coding sequences. In addition, active genes exhibit sites that are at least 1 order of magnitude more sensitive to nucleases than the remainder (Elgin, 1981).

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