High-mobility Group Chromosomal Proteins of Wheat*

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Four proteins have been extracted from purified chromatin of wheat embryos with 0.35 M NaCl. These proteins are soluble in 2% (w/v) trichloroacetic acid and thus meet the original operational requirements to be classified as "high-mobility group" (HMG) chromosomal proteins. The proteins have been characterized by one- and two-dimensional electrophoresis, amino acid analysis, and peptide mapping. Three of the proteins (HMGb, c, and d) share the mammalian HMG characteristic of being rich in both acidic and basic amino acid residues. Unlike their putative mammalian counterparts, these plant HMG proteins contain less than 7 mol % proline. The fourth wheat protein (HMGa) is rich in both proline and in basic amino acid residues. This wheat protein, however, contains only about half the proportion of acidic residues found in mammalian HMG proteins—a characteristic also found in the trout testis HMG protein, H6. Comparative peptide maps show that none of the wheat HMG proteins are degradation products of other HMG proteins or the H1 histones. The peptide maps have not, however, been useful in establishing homologies with mammalian HMG proteins. Wheat HMG proteins are released from DNase I-treated nuclei and co-isolate with micrococcal nuclease-sensitive chromatin fractions. Similar observations concerning the HMG proteins of vertebrate animals have been considered consistent with a role for these proteins as structural components of actively transcribed chromatin.

The HMG1 chromosomal proteins have attracted considerable attention in recent years because, in part, of their postulated roles as structural proteins of nucleosomes of actively transcribed genes (reviewed by Weisbrod, 1982a). Chromatin containing DNA sequences complementary to messenger RNA loses its selective sensitivity to DNase I when extracted with 0.35 M NaCl—a concentration which removes HMG proteins. Moreover, the selective sensitivity can be restored by reconstituting salt-stripped chromatin with the 0.35 M NaCl-extracted proteins or with purified HMG14 or 17 (Weisbrod and Weintraub, 1979; Weisbrod et al., 1980; Gazit et al., 1980). Weisbrod (1982b) and Weisbrod and Weintraub (1981) have demonstrated that an HMG affinity column can selectively retain mononucleosomes which contain DNA complementary to messenger RNA. The manner in which HMG proteins bind to nucleosomes has been studied in many laboratories (see Weisbrod, 1982a and Igo-Kemenes et al., 1982 for review). Because all nucleosomes apparently have two high-affinity HMG binding sites (Mardian et al., 1980; Sandeen et al., 1980), it is uncertain how the postulated specificity in HMG-nucleosome binding might occur. Length of DNA in mononucleosomes isolated in vitro may be involved (Swedlow and Varshavsky, 1983; Stein and Townsend, 1983) but this still leaves open the question of how postulated specific binding may occur in vivo.

The fact that we still do not know the true biological functions of HMG proteins has been highlighted by recent reports which question their long-assumed roles as structural proteins of actively transcribed nucleosomes (Seale et al., 1983; Reeves and Chang, 1983). HMG proteins are, in fact, defined on an operational basis, not on a functional basis (Johns, 1982). The proteins we now call HMG proteins were first noticed as impurities in histone H1 preparations from calf thymus (Johns, 1984). According to the original operational definition presented by Goodwin et al. (1973), HMG proteins are 1) chromatin proteins, 2) extracted from chromatin by 0.35 M NaCl, and 3) soluble in 2% (w/v) trichloroacetic acid. These proteins migrate rapidly in polyacrylamide gel electrophoresis systems; hence, their designation as "high-mobility group" proteins. Further characterization of these proteins revealed certain unusual characteristics which Johns (1982) has added to the operational definition of calf thymus HMG proteins. These are 4) high in basic amino acids—approximately 25 mol %, 5) high in acidic amino acids—approximately 25 mol %, and 6) relatively high in proline—7 mol % or more. The operational definitions of HMG proteins are expedient but, as has been stressed by Johns (1982) and Mayes (1982), care should be taken when such definitions are applied to organisms and tissues other than calf thymus. The operational definitions should aid, not hinder establishing functional definitions for the HMG proteins. An illustration of why the operational definitions cannot be strictly applied is provided by the trout testis protein H6. This protein shows striking sequence homology to calf thymus HMG14 and 17 and is almost certainly the functional equivalent of these mammalian HMG proteins (Dixon, 1982). However, the trout protein does not meet the fifth requirement to be an HMG protein. It contains only about 13 mol % glutamic plus aspartic acid residues.

Proteins that have properties similar to mammalian HMG proteins have been found in many organisms (Mayes, 1982; Bassuk and Mayfield, 1982; Hamana and Iwai, 1979; Katula, 1983; Marquez et al., 1982; Spiker et al., 1978; Weber and Isenberg, 1980). However, the properties of these proteins correspond in varying degrees to those of calf thymus HMG proteins and it is open to question whether they should be considered HMG proteins. We have previously reported the...
existence of four chromosomal proteins from wheat embryos which meet the salt extractability and acid solubility requirements to be termed "HMG" proteins (Spiker, et al., 1978). One of these (wheat HMGb) was additionally shown to be HMG-like in its content of basic and acidic amino acid residues. We have now further characterized the wheat HMG proteins by amino acid composition, molecular weight, peptide maps, and association with putatively transcriptionally active regions of the genome. Comparative amino acid compositions and peptide maps indicate that each of the four plant HMG proteins are genuine proteins and not degradation artifacts of other HMG proteins or H1 histones. Amino acid compositions and peptide maps were not conclusive indicators of which of the plant HMG proteins might correspond to the high-molecular-weight mammalian HMG1 and 2 or the low-molecular-weight mammalian HMG 14 and 17. Wheat HMG proteins b, c, and d meet the requirements for being termed HMG proteins in that they are high in both acidic and basic amino acid residues. However, the proline contents of these are all less than 7 mol %. Wheat HMGa meets the requirement of being relatively high in proline (12 mol %) but, like trout H6, it is relatively low in acidic amino acid residues—about 12 mol %. The plant HMG proteins share the characteristics of vertebrate HMG proteins of being released from nuclei treated with DNase I and co-isolating with the susceptible fraction of chromatin obtained by micrococcal nuclease treatment.

EXPERIMENTAL PROCEDURES

Source of Tissue—Wheat embryos were obtained from General Mills (Minneapolis, MN) or were mechanically isolated from dry, mature grains (Tripplett, 1979). The embryos from both sources yielded chromatin with indistinguishable properties. Thymus glands were obtained from 5-week-old pigs and placed on dry ice within 5 min after slaughter.

Isolation of Chromatin—Chromatin was isolated from wheat embryos by the method of Simon and Becker (1976) with the following modifications. All solutions contained 0.1 mM PhMeSO2F and 12 mM NaHSO3. A Polytron homogenizer (Brinkmann) was used to disrupt the tissue. The final step in the purification was centrifugation of the chromatin through 1.7 M sucrose, 15 mM 2-mercaptoethanol, 10 mM Tris-HCl (pH 8), 0.5% Triton X-100 (Rohm and Hass), 12 mM NaHSO3, 0.1 mM PhMeSO2F for 30 min at 16,000 × g. All procedures were carried out at 0–2 °C.

Isolation of HMG Proteins—HMG proteins were isolated from wheat embryos essentially by the methods used by Goodwin et al. (1971) to isolate calf thymus HMG proteins. These methods are the basis of the operational definition of HMG proteins, i.e. nonhistone proteins extracted from purified chromatin with 0.35 M NaCl and enzized in 0.35 M HCl by

Purification of HMG Proteins—HMG proteins were extracted from frozen pig thymus essentially by the methods used by Goodwin et al. (1977) using nondenaturing pH gradient electrophoresis in the first dimension and SDS gels (as above) in the second dimension.

Isolation of HMG Fractions—Wheat HMG fractions a, b, c, and d were isolated by preparative electrophoresis (Spiker and Isenberg, 1980) or by HPLC. HPLC was carried out using a combination of reverse-phase (Waters Microborepak C-18) and cation exchange (Brownlee Labs CX-300). Details will be presented elsewhere.

Amino Acid Analysis—Amino acid analysis of purified HMG fractions was carried out using the continuous gradient elution method of Klapper (1982).

Peptide Mapping—Peptide maps were obtained by hydrolysis of proteins in gel slices by the method of Cleveland et al. (1977) as modified by Lüna et al. (1979). A further modification of this system (Spiker, 1980) allowed 3-mm-thick gel slices to be placed in 3-mm wells of a stacking gel along with the proteolytic enzymes and the polypeptides to be separated on a 1.5-mm-thick running gel. In the work presented in this paper, a similar modification was used except that the 3-mm-thick stacking gel was poured over a 0.5-mm running gel and a "mini gel" apparatus as described by Matsuda and Burgess (1978) was used.

Salt Extraction of HMG Proteins—The amount of wheat HMG proteins extracted by various concentrations of NaCl was determined as follows. Wheat embryo chromatin purified by centrifugation through 1.7 M sucrose as described above was washed with 75 mM NaCl, 1 mM Tris-HCl (pH 7), 0.1 mM PhMeSO2F. An even suspension of chromatin in this buffer was divided into measured aliquots and adjusted to the desired NaCl concentrations. Each suspension was then layered over a 15% sucrose solution containing the desired NaCl concentration and centrifuged at 47,000 rpm in a Ti 60 rotor (Beckman) for 22 h. The pellets were suspended in water, extracted with 0.4 M H2SO4 and the acid-soluble proteins precipitated by dialysis against ethanol. The supernatants were exhaustively dialyzed against water containing 0.1 mM PhMeSO2F, made 0.4 M H2SO4, and further dialyzed against ethanol to precipitate the proteins. Polyacrylamide-SDS gels were then run, stained with Coomassie Blue and scanned to quantitate DNA-bound protein from the pellets and free proteins from the supernatants.

RESULTS AND DISCUSSION

Electrophoretic Mobility and Molecular Weight Estimation—The electrophoretic mobilities of the wheat HMG proteins are compared to those of mammalian (pig thymus) HMG proteins in the SDS gel in Fig. 1. In general, the electrophoretic mobilities are similar but no wheat HMG matches any pig HMG in mobility.

In Fig. 2 the mobilities of wheat HMG proteins on SDS gels are compared to those of ovalbumin, carbonic anhydrase, myoglobin, cytochrome c, and ubiquitin. Assuming molecular weights of 45,000, 29,000, 17,200, 11,700, and 8,500, respectively, for these standards, the molecular weights of the wheat HMG proteins are calculated to be a = 24,300, b = 20,400, c = 17,400, and d = 14,500. Histones, which have a high proportion of basic residues, migrate anomalously on SDS gels (Panyim and Chalkley, 1971). This anomalous migration leads to an overestimate of their molecular weights when typical molecular weight standards are used for comparison. Like the histones, HMG proteins have a high proportion of basic amino acid residues; but, because they are also high in acidic residues, it is uncertain what standards are appropriate for molecular weight estimation. When pig thymus HMG proteins are used for standards, the molecular weights of the
Wheat HMG Proteins

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gels of wheat embryo and pig thymus HMG proteins. Wheat embryo and pig thymus HMG proteins were isolated and separated by SDSpolyacrylamide gel electrophoresis as described in the text and stained with Coomassie Blue. Pig HMG proteins 1, 2, 14, and 17 are labeled and co-migrate with their counterparts from calf thymus (Goodwin et al., 1978). Contaminating pig thymus H1 histones are also labeled. Wheat HMG proteins a, b, c, and d are labeled.

Fig. 2. Molecular-weight estimation of wheat HMG protein. Sodium dodecyl sulfate-polyacrylamide gels were used as described in the text to electrophoretically separate wheat HMG proteins (left two lanes) and standard proteins of known molecular weights (right two lanes). 1 = ovalbumin, 45,000; 2 = carbonic anhydrase, 29,000; 3 = myoglobin, 17,200; 4 = cytochrome c, 11,700; 5 = ubiquitin, 8,500. Wheat HMG proteins are calculated to be a = 23,100, b = 14,500, c = 10,000, and d = 6,300.

Purification of Fractions—Wheat HMG fractions were purified by preparative electrophoresis (Spiker and Isenberg, 1983) or by HPLC. In the SDS gels in Fig. 3, the purity of the fractions obtained by HPLC is demonstrated. A two-step procedure is used. In the first step, a cation-exchange column at pH 6 and a NaCl gradient separates HMGa and b from HMGc and d. In the second step, a reverse-phase C-18 column separates HMGa from HMGb and HMGc from HMGd. In most cases, no impurities are detected even on overloaded gels. Some contamination of HMGb with HMGc can be noted in panel b of Fig. 3. Scans of Coomassie Blue-stained gels show that HMGb is more than 95% pure. Different preparations of mammalian HMG proteins are contaminated with H1 histones to various degrees (Nicolas and Goodwin, 1982). Such is also the case with wheat HMG preparations which can be seen as the high-molecular-weight band in the unfractionated HMG standards in Fig. 3.

Amino Acid Compositions and Comparison with Calf HMG Proteins—The amino acid compositions of the four calf thymus HMG proteins and the four calf thymus HMG proteins are shown in Table I. The mole per cent of lysine plus arginine is about 20% for each of the wheat HMG proteins. These values are certainly high but not as high as those for the calf thymus HMG proteins. The mole percentages for the acidic residues

<table>
<thead>
<tr>
<th>Residue</th>
<th>Calf thymus mol%</th>
<th>Wheat embryo mol%</th>
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<tr>
<td>Asx</td>
<td>10.7</td>
<td>14.0</td>
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<tr>
<td>Thr</td>
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<td>2.0</td>
</tr>
<tr>
<td>Ser</td>
<td>5.0</td>
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<tr>
<td>Glx</td>
<td>18.1</td>
<td>17.0</td>
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<tr>
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</tr>
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*Goodwin et al. (1978).
for wheat HMGb, c, and d are well within the ranges of those of the calf thymus HMG proteins. Wheat HMGa, however, has only 12.5% acidic residues. This low percentage of acidic residues does not conform to the definitions of HMG proteins proposed by Johns (1982) for calf thymus. However, in light of a similar low percentage of acidic residues in trout testis H6, which is almost certainly an HMG (Dixon, 1982), the possibility that this wheat protein should be considered an HMG protein cannot be ruled out. In fact, if the last characteristic of calf thymus HMG proteins, proline content, is an important diagnostic, HMGa is the only wheat HMG which meets the criterion of at least 7 mol% set by Johns (1982). Wheat HMGa has 12.6 mol% proline. The others have less than 7%.

In order to make a comparison of amino acid compositions of HMG proteins which would take all the amino acids into account, we have used the index of Marchalonis and Weltman (1971) and the critical values of Cornish-Bowden (1980) for testing the significance of amino acid composition indexes. The index, SAQ, is defined as: \(SAQ = 10^4 \left(X_{A} - X_{B}\right)^2\), where \(X_{A}\) is the mole fraction of amino acid residues of the ith type in protein A and \(X_{B}\) is the mole fraction of residues of the ith type in protein B. Cornish-Bowden (1980) has developed tables of "critical values" for predicting "relatedness" of proteins based on amino acid composition comparison by \(SAQ\). The values are dependent upon the total number of amino acid residues in the larger of the two proteins (N). For each N, critical values for a "strong" test and for a "weak" test have been calculated. According to Cornish-Bowden, the calculated value of \(SAQ\) nearly always will be greater than the critical value for the "strong" test if the proteins are unrelated. Calculated values of \(SAQ\) will almost always be smaller than the critical value for the "weak" test if the proteins are related.

We have calculated \(SAQ\) for all pairs of wheat and calf HMG proteins and compared the values to the critical values of Cornish-Bowden (1980) in an attempt to determine if any of the wheat HMG proteins are more closely related to the larger calf HMG proteins (1 and 2) or to the smaller calf HMG proteins (14 and 17). The results of these calculations are summarized in Fig. 4. In this figure if two proteins meet the "weak" test requirement for being related, they are connected by a dotted line. If they meet the "strong" test for being related, they are connected by a solid line. It should be emphasized that we make no claims that proteins are necessarily related by sequence if they meet the "weak" or "strong" test for relatedness. Nor do we claim that proteins which do not meet the "weak" test are necessarily unrelated. For example, calf HMG14 and HMG17 have been entirely sequenced (Walker et al., 1977, 1979), and even though they do not meet even the "weak" test for relatedness, they are obviously related by sequence. The sequences of these two proteins can be aligned with two gaps in each protein resulting in a 44% sequence homology. This homology includes a stretch in which no gaps are necessary to generate 12 consecutive and 19 of 22 sequence identities. Conversely, calf thymus HMG2 and HMG14 meet the "weak" test for relatedness even though there is little sequence homology between the two proteins.

Calf HMG2 (Walker, 1982) and HMG14 contain only two common sequences with as many as four consecutive amino acid residues.

As can be seen in Fig. 4, wheat HMGb and HMGd meet the "strong" test criterion for being related. Wheat HMGc meets the "weak" test criterion for being related to wheat HMGb and HMGd. Wheat HMGc and HMGd meet the "weak" test for being related to calf thymus HMG14. No other relationships are evident using the criteria of Cornish-Bowden (1980).

Wheat HMGa has some characteristics reminiscent of H1 histones. It has high proline and alanine content. However, it is much lower in lysine and higher in arginine than wheat H1 histones (Spiker et al., 1976). No sequences are available for wheat H1 histones. However, we have used the partial sequence information and amino acid compositions of amino- and carboxy-terminal regions of maize H1 histone (Hurley and Stout, 1980) to determine if the amino acid composition of HMGa could be consistent with that of a simple degradation product of H1 histone. Using this approach, we could find no evidence that HMGa is derived from H1. Comparative peptide maps of wheat HMGa and total H1 histones (data not shown) also provide no evidence that HMGa is derived from H1. When the amino acid compositions of HMGa and total H1 histone are compared by the method of Cornish-Bowden (1980), the two proteins do not meet the "weak" test for being related. By the same procedures, none of the wheat HMG proteins have amino acid compositions which are "related" to any of the wheat H2 histones.

**Two-dimensional Electrophoresis**—Commercially available ampholines have not proved useful in characterizing the wheat HMG proteins by isoelectric focusing. Thus, we have used nonequilibrium pH gradient electrophoresis (O'Farrell et al., 1977). In Fig. 5, a two-dimensional gel is shown in which a
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Wheat HMG proteins are separated by nonequilibrium pH gradient electrophoresis in the first dimension and SDS electrophoresis in the second dimension. HMGs and contaminating histone H1 are labeled in the figure. Calf thymus HMG proteins have secondary modifications and thus are separated into multiple forms by isoelectric focusing (Nicolas and Goodwin, 1982). Similarly, wheat HMGb has two forms—one which migrates slowly in the first dimension and one which migrates nearly as rapidly as HMGa. HMGc and d also have two forms each when separated by this procedure.

Peptide Mapping—We have used the procedures of Cleveland et al. (1977) to compare the peptide maps of each of the wheat HMG proteins with the others, with each of the pig thymus HMG proteins, and with wheat H1 histones. Some of the maps are shown in Fig. 6. When the maps of the wheat HMG proteins are compared, a few bands of common mobility can be demonstrated. However, there is no indication that any of the lower-molecular-weight HMG proteins are derived from any of the higher-molecular-weight ones or that extensive sequence homology exists in the wheat HMG proteins. Similarly, in comparison of wheat HMG maps with those of the pig thymus HMG proteins, no evidence of extensive homology can be found.

Salt Extraction of Wheat HMG Proteins—We have used extraction of purified wheat chromatin with 0.35 M NaCl in order to establish that the proteins we have studied meet the original operational criteria for HMG proteins. However, we notice that when such procedures are used, some of the HMG proteins are not extracted from chromatin. In Fig. 7, the percentages of HMG proteins extracted by various concentrations of NaCl are shown. Well over half of the total HMG proteins are extracted by 0.35 M NaCl. However, about 10% of the HMG proteins remained bound to chromatin at 0.4 M NaCl and all detectable HMG proteins were not removed until 0.45 M NaCl.

Association with Putatively Active Chromatin—Some of the first, but certainly not conclusive, indications that vertebrate HMG proteins are structural proteins of active chromatin were the observations that these proteins are released from DNase I-treated chromatin (Vidali et al., 1977; Levy-Wilson et al., 1977). We have previously shown (Spiker et al. 1983) that DNase I treatment of nuclei releases the wheat proteins which we have called HMG proteins. Micrococcal nuclease also more readily attacks chromatin containing transcribed sequences than it attacks bulk DNA (Bloom and Anderson, 1978). Jackson et al. (1979) have developed a procedure which uses micrococcal nuclease to obtain a chromatin fraction which contains HMG proteins in amounts stoichiometric with the histone fractions. When the procedure of Jackson et al. (1979) is used on wheat chromatin, fractions similarly highly enriched in the proteins we call HMG proteins are obtained. Fig. 8 shows SDS gels of proteins of the wheat chromatin fractions prepared by the procedures of Jackson et al. (1979). In lane 1 of this figure are the proteins of the micrococcal nuclease-resistant fraction. The only prominent proteins in this fraction are the histones. In lane 2 of this figure are the proteins of the micrococcal nuclease-susceptible fraction. This fraction contains histones but it also contains very prominent proteins which co-migrate with the wheat proteins we have called HMG proteins. This fraction also contains a number of higher-molecular-weight proteins which may be analogous to the proteins studied by Prior et al. (1983) or by Emerson and Felsenfeld (1984).
weight of this observation must be considered in light of similar acidic amino acid residue content of trout testis protein H6 which, on the basis of amino acid sequence, is almost certainly an HMG protein (Dixon, 1982). The wheat HMG which is uncharacteristically low in acidic amino acid residues, HMGa, is the only wheat HMG which meets the proline-content criterion suggested by Johns (1982) for calf thymus HMG proteins. Wheat HMGa contains about 13 mol \% proline residues. The other three wheat HMG proteins contain 4–6 mol \%. We were unable to assign any of the plant HMG proteins to the larger HMG class (corresponding to calf thymus HMG1 and 2) or to the smaller HMG class (corresponding to calf thymus HMG14 and 17) either by simple inspection of the amino acid contents or by using the procedures of Cornish-Bowden (1980). Wheat HMGc and HMGd do, however, meet the "weak" requirement of Cornish-Bowden for being "related" to calf thymus HMG14. Comparative peptide mapping also failed to establish extensive homology between any of the plant and vertebrate HMG proteins. The results of the mapping studies did, however, indicate that the wheat HMG proteins were not degradation products of higher-molecular-weight HMG proteins or of the H1 histones.

The same HMG proteins which are extracted from wheat embryo purified chromatin can be extracted from various differentiated tissues of wheat with 0.5 N HClO4, and these proteins are associated with mononucleosomes separated on sucrose gradients (data not shown). The observation that wheat HMG proteins are associated with DNase I- and micrococcal nuclease-sensitive chromatin is consistent with the idea that they are the functional equivalents of mammalian HMG proteins. Complete sequence data on the plant HMG proteins would be helpful in establishing such a correspondence. However, it is obvious that correspondence in function cannot be reliably established until the true in vivo functions of mammalian HMG proteins and plant HMG proteins are established.

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REFERENCES

Summary—Four proteins are extracted from purified wheat embryo chromatin with 0.35 M NaCl and are soluble in 2% (w/v) trichloroacetic acid. Thus, these proteins meet the original operational criteria for being "HMG" proteins. Because the true biological roles of mammalian HMG proteins have not been established, we cannot as yet determine if the plant HMG proteins are the functional equivalents of mammalian HMG proteins. The plant HMG proteins do have several characteristics, however, which are consistent with the idea that they do have the same role in chromatin structure as mammalian HMG proteins. They are rich in basic amino acid residues—approximately 20 mol \%. Three are rich in acidic amino acid residues—approximately 23–29 mol \%. The fourth wheat HMG has only about 12 mol \% asparagine and glutamate + glutamine residues. Thus, this protein is uncharacteristically low in acidic residues. The
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