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Detection of mRNAs of Carcinoembryonic Antigen and Nonspecific Cross-reacting Antigen Genes in Colorectal Adenomas and Carcinomas by *in situ* Hybridization

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Expression of mRNAs of carcinoembryonic antigen (CEA) and nonspecific cross-reacting antigen (NCA) genes in colorectal carcinomas and adenomas was investigated by *in situ* hybridization (ISH) with specific biotinylated probes. Hybridization was clearly detected throughout the cytoplasm of 7 out of 15 adenomas and 13 out of 15 carcinomas with the CEA cDNA probe, and in 6 out of 15 adenomas and 10 out of 15 carcinomas with the NCA cDNA probe. The intensity of signal appeared to be stronger in carcinomas than that in adenomas, and the CEA and NCA mRNAs were expressed together in most of the positive tissue specimens. On the other hand, noninvaded tissues adjacent to the carcinoma did not show any signal except for 4 cases faintly stained with the NCA probe. This finding was partly confirmed by Northern blot analysis which indicated that the specific bands for the CEA and NCA mRNAs were more intense in RNAs from carcinoma tissues than in those from adjacent noninvaded tissues. These data suggest that the ISH technique with biotinylated probes could be of use for analyzing expression and localization of CEA and related genes on tissue sections.

Key words: *In situ* hybridization — Carcinoembryonic antigen — Nonspecific cross-reacting antigen — Colorectal carcinoma

Carcinoembryonic antigen (CEA)[2] is the most useful tumor marker for monitoring patients with colorectal carcinomas. However, the existence of many serologically cross-reacting antigens has made complicated the analysis of this molecule[1] even though great efforts have been made with monoclonal antibodies.[2] Recent molecular biological and biochemical approaches have demonstrated that CEA and related antigens belong to a gene family consisting of at least 11 related genes.[3–9] The nucleotide sequencing of these cDNAs revealed a specific sequence for each gene, now making it possible for us to distinguish each member of the CEA gene family by using these sequences as probes at the level of mRNA.[10–12]

The *in situ* hybridization (ISH) technique has been widely used for detection of mRNAs in addition to Northern blot analysis.[13] Rather than analyzing an RNA after its extraction from a heterogeneous cell population, detection of mRNA *in situ* identifies the localization of specific sequences within a tissue. This will make it possible to identify the morphological characteristics of the cells expressing specific mRNAs. We therefore examined the expression of the CEA and nonspecific cross-reacting antigen (NCA) mRNAs in colorectal adenomas and carcinomas by ISH with specific probes. This is the first analytical report on an application of ISH to the CEA gene family.

MATERIALS AND METHODS

Cultured cell and tissue preparations Human colonic cancer cell line BM314 (kindly donated by Dr. W. A. Nelson-Ree, Univ. of California, Berkeley, CA) and gastric cancer cell line MKN45 for CEA-producing cell lines and human hepatocellular cell line cHc-4 (Dr. J. Uchino, Univ. of Hokkaido, Sapporo) for a CEA nonproducing cell line were cultured in RPMI1640/10% fetal calf serum on microscope slides. After being washed several times with RPMI1640, the cells were fixed in 4% paraformaldehyde/PBS, pH 7.5.

Fifteen colorectal adenocarcinomas and adjacent noninvaded tissues used for ISH were surgically resected and immediately fixed in 4% paraformaldehyde/PBS, pH 7.5. Fifteen colorectal polyps were obtained by polypectomy with a fiberscope and were fixed in the same manner. These tissues were dehydrated in 30% sucrose, embedded in OCT compound (Miles, IN) and then frozen at −80°C.

***In situ* hybridization** ISH was performed according to Singer *et al.*[13] with some modifications. Frozen sections were cut at 5 μm and mounted on microscope slides. The sections were hydrated in PBS, pH 7.5 for 10 min and

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[2] Abbreviations used are: CEA, carcinoembryonic antigen; NCA, nonspecific cross-reacting antigen; ISH, *in situ* hybridization; mRNA, messenger RNA; cDNA, complementary DNA; PBS, phosphate-buffered saline; MAb, monoclonal antibody.

then incubated in 0.01 *M* EDTA/0.01 *M* $MgCl_2$ for 30 min to inhibit internal alkaline phosphatase as described by Jensen *et al.*[14] The slides were incubated in 0.2 *M* Tris-HCl, pH 7.5/0.1 *M* glycine for 10 min, and transferred to 2×SSC/50% deionized formamide at 70°C just prior to hybridization. A 0.39 kb *Rsa*I/*Eco*RI fragment from the CEA 3′-untranslated region cDNA and a 0.85 kb *Eco*RI/*Hin*dIII fragment from the NCA 3′-untranslated region cDNA (gift of Dr. J. E. Shively, Beckman Research Institute of the City of Hope, Duarte, CA) were labeled with biotin 11-dUTP (Bethesda Research Laboratories, MD) according to Feinberg *et al.*[15] The heat-denatured labeled probe (0.5 μg/ml) was mixed with hybridization mixture containing 50% deionized formamide, 1 mg/ml of *E. coli* tRNA (Sigma, MO), 1 mg/ml of heated salmon sperm DNA, 2×SSC, 0.2% bovine serum albumin, 10% dextran sulfate (Pharmacia, Sweden) and 20 m*M* vanadyl ribonucleoside complex (Bethesda Research Laboratories) and was applied in 20 μl amounts to each slide. Hybridization was carried out in a humidified chamber at 37°C for 16 h. The unhybridized probe was washed off in 50% formamide/2× SSC at room temperature for 30 min, in 50% formamide/1×SSC, for 30 min and finally in 1×SSC for 30 min. The slides were washed, incubated at 37°C for 20 min in a solution containing buffer A (0.1 *M* Tris-HCl, pH 7.5/0.1 *M* NaCl/2 m*M* $MgCl_2$/0.5% Triton X-100) and 3% bovine serum albumin, and then placed in a solution of 1 μg/ml of streptavidin (Sigma) in buffer A for 1 h. After being washed in buffer A for 20 min with a buffer change every 5 min, they were incubated with 1 U/ml of biotinylated alkaline phosphate in buffer A for 1 h. The slides were washed with two changes of buffer A and given a final rinse with buffer B (0.1 *M* Tris-HCl, pH 9.5/0.1 *M* NaCl/5m*M* $MgCl_2$) for 10 min with two changes. The slides were put in a solution with 330 μg/ml nitroblue tetrazolium (Sigma) and 167 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) for 3–12 h to detect the signals. The sections were counterstained with methylgreen.

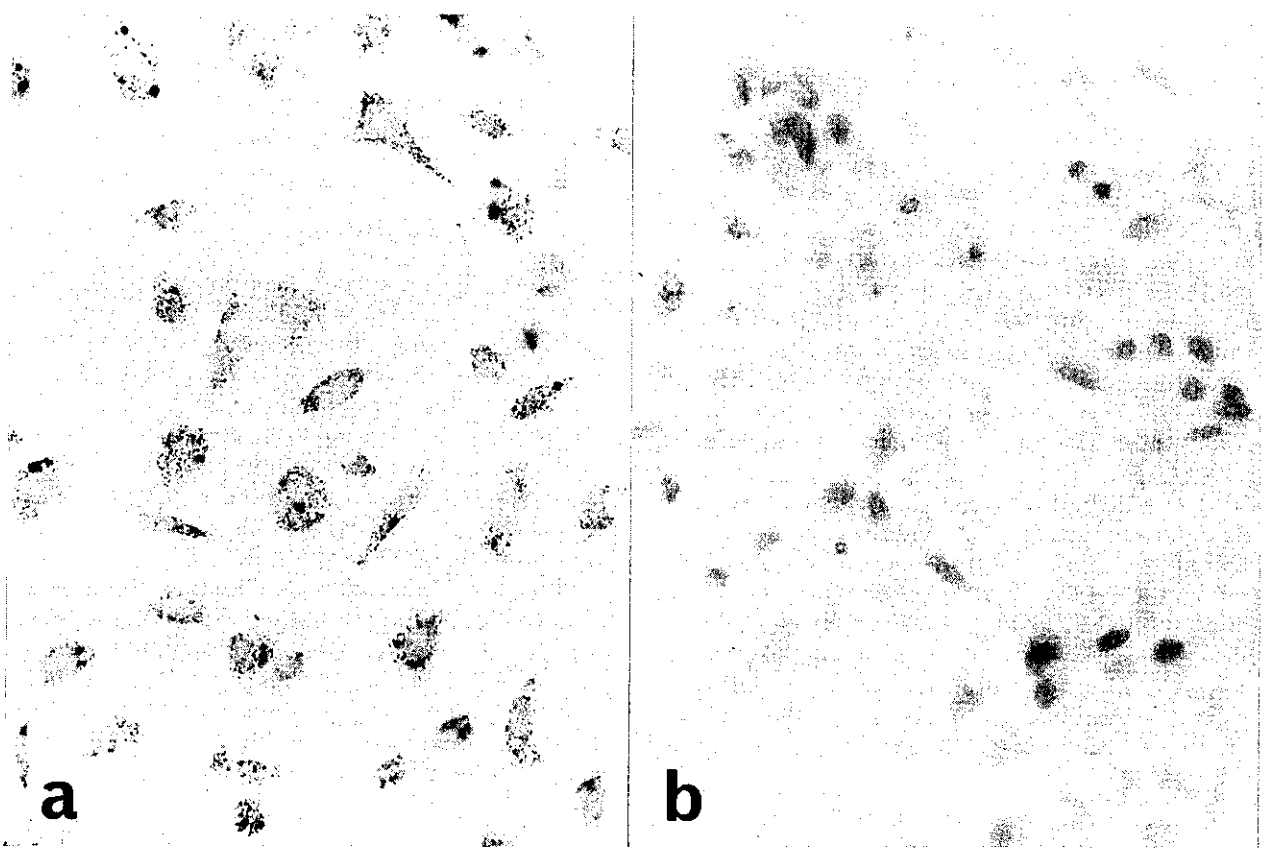

Fig. 1. *In situ* hybridization of cultured carcinoma cells with a biotinylated cDNA probe specific for the CEA gene. CEA-producing colon cancer cell line BM314 (a) was positive, whereas CEA-nonproducing hepatoma cell line cHc-4 (b) was negative for the probe. (×470).

Northern blot analysis Total RNAs (10 μg) prepared from tissues of colorectal adenomas, carcinomas, and adjacent noninvaded tissues as described[7] were electrophoresed through 1% agarose gel containing 16% formaldehyde and Goldberg buffer, transferred to nitrocellulose filters, and hybridized with a cDNA probe labeled by random priming[15] at 42°C for 20 h. After being washed at a final stringency of 0.1×SSC/0.1% SDS at 60°C for 30 min, filters were exposed to Hyperfilm-MP (Amersham, Sweden).

RESULTS

To assess the specificity of the reaction, some experiments were carried out and the following results were obtained. First, the hybridization signal was detected in CEA-producing cell lines, not in nonproducing ones, with the 3′-untranslated region probe from cDNA of CEA (Fig. 1). Secondly, the treatment of CEA-producing cell line BM314 with RNaseA or an unlabeled 3′-untranslated region probe clearly diminished the intensity of the hybridization signal. Finally, hybridization of BM314 cells was performed with the probe from cDNA of the human chorionic gonadotropin gene to detect nonspecific binding, showing no hybridization signal (data not shown). These data demonstrated that the specific mRNA for CEA could be detected by a biotinylated cDNA probe. We also examined the specificity of the reaction in the same manner on colorectal carcinoma tissue sections. Although the results were reproducible, nonspecific binding of tissue sections was a little higher compared to that of BM314 cells (data not shown), which was in accordance with the report by Singer *et al.*[13]

As shown in Table I, the hybridization signals for the CEA and NCA mRNAs were detected in 12 and 10 out of 15 colorectal carcinoma tissue specimens, respectively, with specific biotinylated probes. mRNAs of CEA and NCA were present throughout the cytoplasm of carcinoma cells and their localizations were almost identical

Table I. *In situ* Hybridization Analysis of Colorectal Carcinomas

Case No.	Histology	*In situ* hybridization[a]			
		CEA		NCA	
		Ca[b]	N[c]	Ca	N
C1	mod. diff. adenoca.[d]	++	−	+	−
C2	mod. diff. adenoca.	++	−	+	−
C3	mod. diff. adenoca.	++	−	+	−
C4	mod. diff. adenoca.	++	−	++	−
C5	mod. diff. adenoca.	++	−	++	−
C6	mod. diff. adenoca.	++	−	+/−	+/−
C7	mod. diff. adenoca.	++	nt[g]	+	nt
C8	mod. diff. adenoca.	+	−	+	+/−
C9	mod. diff. adenoca.	+/−	−	−	−
C10	mod. diff. adenoca.	+/−	−	−	−
C11	mod. diff. adenoca.	−	−	+/−	+/−
C12	mod. diff. adenoca.	−	−	−	−
C13	mod. diff. adenoca.	−	−	−	−
C14	poorly diff. adenoca.[e]	+/−	−	++	+/−
C15	mucinous ca.[f]	+/−	−	−	−

a) Intensity of *in situ* hybridization was scored as negative (−), slightly positive (+/−), positive (+) or strongly positive (++).
b) Ca, colonic carcinoma.
c) N, adjacent normal tissue.
d) mod. diff. adenoca., moderately differentiated adenocarcinoma.
e) poorly diff. adenoca., poorly differentiated adenocarcinoma.
f) mucinous ca., mucinous carcinoma.
g) nt, not tested.

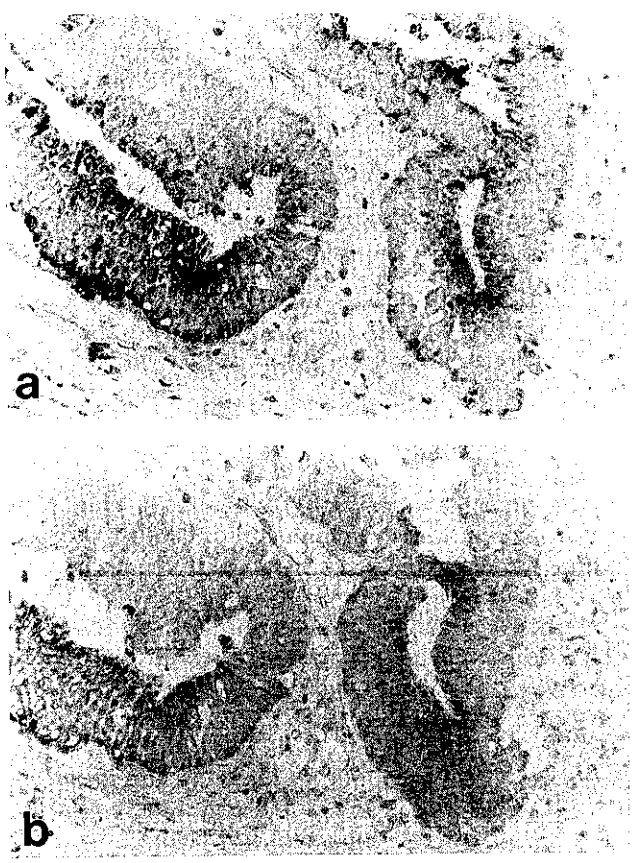

Fig. 2. Detection of mRNAs of the CEA and NCA genes on colorectal carcinoma tissue sections. Fifteen tissue samples were tested. Expression of the CEA mRNA (a) and the NCA mRNA (b) was detected in the cytoplasm of carcinoma cells. (×340).

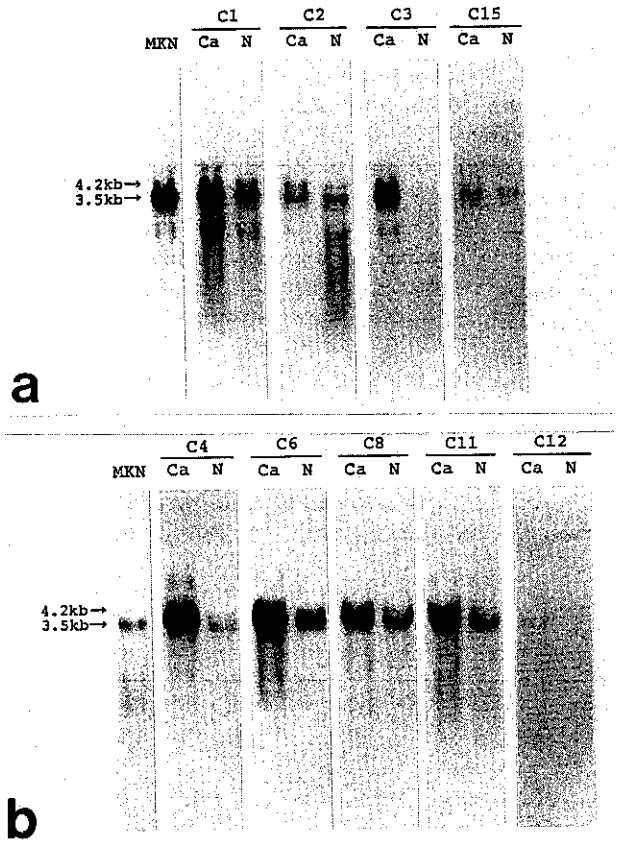

Fig. 3. Northern blot analysis of total RNA (10 μg) from colorectal carcinoma and adjacent normal tissues with a ^{32}P-labeled CEA cDNA probe. The results of two experiments (a, b) are shown. Case numbers are indicated at the top of the figures. MKN is a human gastric cancer cell line MKN45. Ca and N indicate the carcinoma tissue and the adjacent normal tissue, respectively.

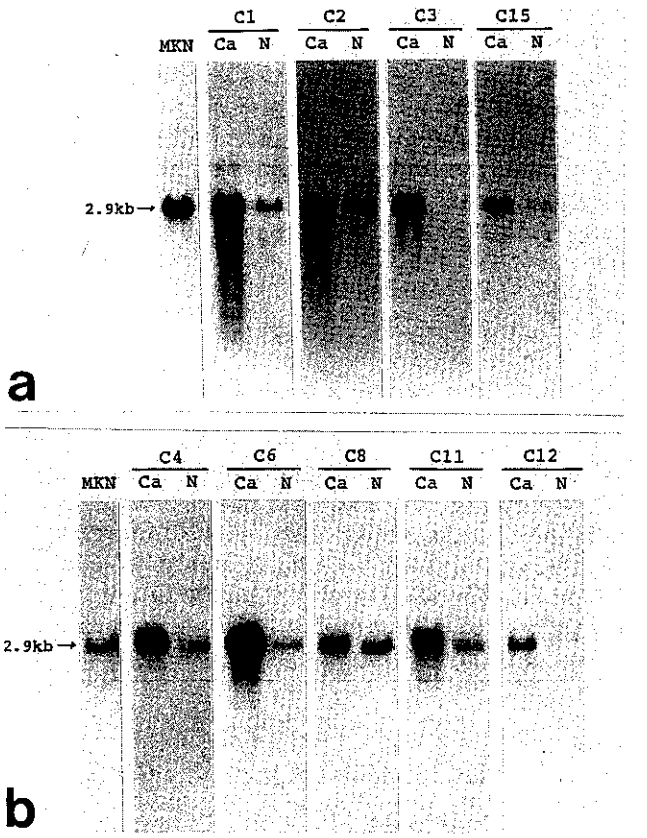

Fig. 4. Northern blot analysis of total RNA (10 μg) from colorectal carcinoma and adjacent normal tissues with a ^{32}P-labeled NCA cDNA probe. The results of two experiments (a, b) are shown. Case numbers are indicated at the top of the figures. MKN is a human gastric cancer cell line MKN45. Ca and N indicate the carcinoma tissue and the adjacent normal tissue, respectively.

in carcinoma tissues (Fig. 2), although 4 noninvaded tissues adjacent to the carcinoma showed a weak hybridization signal for the NCA mRNA. It should be noted that both messages were expressed together in 9 tissue specimens. Although recent reports[16,17] have demonstrated that NCA-like antigens exist in peripheral blood leucocytes, they could not be clearly distinguished from nonspecific signals in our present experiments.

Northern blot analysis was then performed to confirm the expression of these mRNAs in tissue specimens used for ISH. As shown in Fig. 3 and Fig. 4, both CEA and NCA-specific bands including 3.5 kb and 4.2 kb for the CEA mRNA and 2.9 kb for the NCA mRNA[10] were detected in 9 specimens. The intensity of the bands was consistently stronger in carcinomas than in adjacent noninvaded tissues, especially for the NCA mRNA, which was consistent with previous reports.[10,12] Since experiments a and b in Fig. 3 and Fig. 4 were independently carried out, the intensities of hybridization signals are not directly comparable.

The data on colorectal adenomas in Table II indicate that mRNAs of CEA and NCA were detected by ISH in 7 and 6 out of 15 tissue specimens, respectively, and were expressed together in 5 specimens. Also in the case of adenomas, the cytoplasm was diffusely stained and no significant difference was observed in the localization of mRNA between CEA and NCA. Thus, it appears that expression of the CEA and NCA mRNAs was lower in adenomas, compared to carcinomas.

DISCUSSION

The *in situ* hybridization (ISH) technique with nonisotopic probes has recently been developed to overcome

Table II. *In situ* Hybridization Analysis of Colorectal Adenomas

Case No.	Histology	*In situ* hybridization[a]	
		CEA	NCA
A1	tubulo-villous adenoma	+	+
A2	tubulo-villous adenoma	+	+/−
A3	tubular adenoma	+	−
A4	tubular adenoma	+/−	+
A5	tubular adenoma	+/−	+/−
A6	tubular adenoma	+/−	+/−
A7	tubular adenoma	+/−	−
A8	tubular adenoma	−	+/−
A9	tubular adenoma	−	−
A10	tubular adenoma	−	−
A11	tubular adenoma	−	−
A12	tubular adenoma	−	−
A13	tubular adenoma	−	−
A14	hyperplastic polyp	−	−
A15	hyperplastic polyp	−	−

a) Intensity of *in situ* hybridization was scored as negative (−), slightly positive (+/−), or positive (+).

some disadvantages of radioisotopic probes, such as decay of the radiolabel, poor resolution on autoradiography, the long time required for detection of the signal and general safety considerations. We therefore used here biotinylated cDNAs as probes for ISH to detect the mRNAs of CEA and NCA in tissue sections fixed with paraformaldehyde according to the method of Singer *et al.*[13] The streptavidin-alkaline phosphatase system used in conjunction with biotins was considered to be convenient and compatible with clinical laboratory practice, although this procedure is not as sensitive as autoradiography at the present time.[13] When the results of RNA blot analysis with isotopic probes were compared to those of ISH, sensitivity seemed to be superior in the former, because specific bands for the CEA mRNA were detected by Northern blot analysis in RNA from noninvaded tissues (Fig. 3) despite the fact that almost no signal was observed on the tissue sections by ISH (Table I). Thus, it appears that the most important advantage of the ISH technique is to give morphological information specific for each gene.

The present data show that mRNAs of CEA and NCA were present throughout the cytoplasm of adenoma and carcinoma cells of the colon. Recently, similar results on CEA have been reported, which indicated that the sulfonated cDNA probe could detect mRNA of CEA in normal colon epithelial cell.[18] Although their procedures for labeling probes and fixing tissues were different from ours, it can not be completely excluded that their cDNA probe encoding a part of the N-terminus and immunoglobulin-like domain may cross-react with the NCA and BGP I mRNAs because of their high sequence similarity.[3, 5, 7] It was also shown that intensity of staining and localization of the CEA and NCA mRNAs in each tissue section were very similar to each other, suggesting that transcription of these two genes might be regulated by some common mechanisms. When the intensity of the bands in RNA blot analysis was compared between carcinomas and adjacent noninvaded tissues, expression of CEA and NCA mRNAs appeared to be more prominent in the former than in the latter. This was in keeping with the results in previous reports,[10, 12] suggesting that NCA as well as CEA could be of use as a tumor marker for colorectal carcinoma. The quantitative analysis of the NCA mRNA and the development of NCA-specific monoclonal antibodies will be required to confirm this. With regard to adenomas, the present data obtained by ISH suggested that mRNAs of CEA and NCA are expressed with an intensity intermediate between the levels in normal and carcinoma tissues. Recent findings on the chromosomal abnormalities and the point mutations of K-*ras* oncogene have strongly supported the hypothesis that colorectal adenomas could be a precancerous lesion of the colon.[19, 20] Further studies will be required to determine whether transcription of the CEA and NCA genes can be related with those gene abnormalities or not.

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