[CANCER RESEARCH 52, 2384–2388, May 1, 1992]

Retinoic Acid Inhibition of Serum-induced c-*fos* Transcription in a Fibrosarcoma Cell Line[1]

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ABSTRACT

We investigated the mechanism by which retinoic acid causes growth arrest and flat reversion of SSV-NRK, simian sarcoma virus-transformed normal rat kidney cells. Northern analysis revealed that both chronic (7 days) and acute (6 h) retinoic acid treatment of serum-stimulated SSV-NRK cells caused a 6-fold decrease in c-*fos* mRNA levels. In addition, nuclear run-on experiments showed that retinoic acid regulated c-*fos* expression in SSV-NRK cells at the transcriptional initiation level. Attenuation of c-*fos* transcription was equal in both retinoic acid-treated and control cells, and no increased c-*fos* mRNA turnover was detected in retinoic acid-treated cells. Furthermore, there was no observed change in the c-*fos* mRNA levels after only 30 min of retinoic acid treatment, suggesting that a mechanism involving the interruption of the signal transduction mechanism at the membrane level is unlikely. Because it has been shown that c-*fos* expression plays a pivotal role in mitogenesis of quiescent fibroblasts, we conclude that the retinoic acid-mediated down-regulation of c-*fos* expression is a mechanism for growth inhibition in SSV-NRK cells.

INTRODUCTION

In 1925 it was initially observed in rats that vitamin A deficiency leads to squamous metaplasia of mucous membranes and hyperkeratosis of the skin (1). Since then, RA,[3] a physiologically active vitamin A derivative, has been shown to inhibit the proliferation of a broad range of transformed cell types (2–4), as well as to induce benign (terminal) differentiation in a group of transformed cell lines, including F9 murine teratocarcinoma (5), SMS-KCNR human neuroblastoma (6), and HL-60 human promyelocytic leukemia (7). The mechanism by which RA mediates its multiple effects on transformed cells is not well understood.

RA has been shown to inhibit anchorage-independent growth of SSV-NRK, a rat fibroblast cell line transformed by simian sarcoma virus (8). Autocrine stimulation of these cells by $p28^{sis}$, a homologue of platelet-derived growth factor encoded by v-*sis*, resulted in unregulated proliferation (9). Although constitutive expression of v-*sis* has been demonstrated to be essential for the transformed state of the cells (10), RA has been shown to have no effects on v-*sis* expression (8). Therefore, other mechanisms are probably involved in mediating the growth inhibition of the transformed cells by the retinoids.

A cascade of events occurs following mitogenic stimulation of fibroblasts by growth factors, leading eventually to cell division. Belonging to a group of immediate-early genes, c-*fos* plays a pivotal role in these events, allowing cells to pass from G_0-G_1 to the S phase of the cell cycle (11). Mitogenic stimulation of serum-starved fibroblasts by serum or growth factors such as platelet-derived growth factor is associated with a very rapid induction of c-*fos* (12, 13). When a plasmid expressing antisense c-*fos* RNA is introduced into such an experimental system to block c-*fos* expression, the cell division does not occur (14). Furthermore, transformed cells expressing antisense c-*fos* undergo flat reversion suggestive of benign differentiation of the neoplastic cells (15). We observed that RA also caused flat reversion of SSV-NRK cells. Therefore, we hypothesize that RA affects the signal transduction pathway, in particular the expression of c-*fos*. In this paper, data will be presented showing down-regulation of c-*fos* transcription in serum-stimulated SSV-NRK cells by RA.

MATERIALS AND METHODS

Cell Culture. SSV-NRK cells (a gift from Dr. S. Aaronson) were grown in Dulbecco's medium enriched with 10% heat-inactivated calf serum. For treatment of cells, a stock of 4×10^{-3} M all-*trans*-RA (Sigma, St. Louis, MO) was prepared in 100% ethanol, stored at −20°C, and diluted immediately before use. In control cultures, ethanol was added to a final concentration of 0.25%. Cycloheximide was added to 50 μg/ml where indicated. Cell growth kinetics were measured in triplicate in 35-mm plates seeded with 2×10^4 cells/plate and cell number was counted daily with a hemocytometer. Routine tests for *Mycoplasma* by the Hoechst staining method (16) were negative.

RNA Extraction. RNA was prepared by the method of Chirgwin *et al.* (17). Briefly, total cellular RNA was extracted from approximately 10^7 SSV-NRK cells. Cells were lysed with 6.7 M guanidine isothiocyanate (Research Organics, Cleveland, OH) in the presence of 1 mM vanadyl ribonucleoside complex (Bethesda Research Laboratory, Gaithersburg, MD), a RNase inhibitor. The lysate was centrifuged over a cushion of 3.5 M CsCl. The pelleted RNA was dissolved in water.

Gel Electrophoresis of RNA and Northern Transfer. Twenty μg denatured total RNA was fractionated by electrophoresis on agarose gel by using 10 mM NaH_2PO_4 buffer, pH 7.2 (18). An RNA ladder (Bethesda Research Laboratory) was included as size standard. RNA was transferred to nylon membrane (Bio-Rad, Richmond, CA) by an alkaline transfer method of Vrati *et al.* (19). After transfer was completed, the membrane was rinsed in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 0.1% SDS and allowed to dry. It was then baked at 80°C in a vacuum oven for 1 h.

Hybridization. Prehybridization, hybridization, and washing were carried out under conditions specified by Schleicher & Schuell (Keene, NH). Prehybridization and hybridization were carried out in solutions consisting of 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50% formamide, 0.1% SDS, 5 × SSPE (1 × SSPE is 0.18 M NaCl, 0.01 M $NaPO_4$, pH 7.6, 1 mM EDTA), and 250 μg/ml denatured herring sperm DNA at 42°C for 6–24 h and 24–36 h, respectively. v-*sis*, v-*fos*, c-*myc* (Oncor, Gaithersburg, MD), and c-*jun* (20) probes as well as rat α-tubulin (21) were labeled with [α^{32}-P]dCTP (ICN, Irvine, CA) by the oligolabeling method (Pharmacia Laboratories, Piscataway, NJ) to at least 1×10^8 cpm/μg DNA. After hybridization, the filters were washed sequentially in 6 × SSPE and 0.5% SDS at room temperature for 15 min twice, in 1 × SSPE and 0.5% SDS at 37°C twice for 15 min, and

Received 9/27/91; accepted 2/20/92.

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[1] This work was supported by USPHS Grant ES04132 to L-N. L. C. and USPHS Grant GM27589 to T-s. C. P. J. is the recipient of a McLaughlin postdoctoral fellowship.

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[3] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; SRE, serum response element; SRF, serum response factor; SSC, standard saline citrate; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; SSPE, saline-sodium phosphate-EDTA.

in the latter solution at 65°C for 45 min. The filters were then exposed to X-ray film, using an intensifying screen at −70°C.

Nuclear Run-On Transcription. Run-on transcription was carried out as described by Mahajan and Thompson (22) with modifications. Nuclei were prepared from approximately 10^7 cells, and *in vitro* transcription was initiated by adding to 10 μl nuclear suspension a 100-μl mixture of 0.5 M Tris-Cl, pH 7.8, 0.25 M NaCl, 1.75 M $(NH_4)_2SO_4$, 0.01 M EDTA, 0.2 M $MnCl_2$, 1 mg/ml heparin, and 100 μM each of ribonucleoside triphosphates containing 100 μCi [α-^{32}P]UTP (Du Pont NEN, Wilmington, DE) at 32°C. After incubation for 45 min, RNA was extracted with phenol/chloroform, and ethanol precipitated. Nuclear equivalents of labeled RNA from control and RA-treated cells were hybridized to M13 DNA probes containing either exon 1 or exons 3 and 4 of murine c-*fos* (23) that were immobilized on nitrocellulose filters by using the slot-blot apparatus (Schleicher & Schuell). Plasmid pTh1 (kindly provided by Dr. E. A. Thompson), which encodes 5S RNA, was used as positive control. Prehybridization and hybridization were carried out in a buffer containing 0.01 M NaCl, Denhardt's solution, and 100 μg/ml polyadenylic acid at 65°C for 2 and 40 h, respectively. Filters were washed sequentially with 2 × SSC at 65°C for 1 h, 2 × SSC containing 10 μg/ml RNase A (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 min, and 2 × SSC at 37°C for 1 h. The filters were then exposed to X-ray film as described above.

Materials. Ultrapure cesium chloride was from Bethesda Research Laboratories. Agarose (Seakem LE) was supplied by FMC BioProducts (Rockland, ME). Polyadenylic acid was purchased from Boehringer Mannheim. Actinomycin D and cycloheximide were supplied by Merck Sharp & Dohme (West Point, PA) and Sigma, respectively. All other reagents were of the highest grade available and were purchased from standard sources.

Fig. 1. Northern blot analysis of c-*fos* (*A*) and v-*sis* (*B*) mRNA in RA-treated SSV-NRK cells. The cells were grown to confluence, serum starved, and serum stimulated as described in the text. Cells were treated with 10^{-5} M RA (*Lane 1*) or untreated (*Lane 2*). The sizes of c-*fos* and v-*sis* mRNA are 2.2 and 2.7 kilobases (*kb*), respectively. The same blots were stripped and rehybridized with a tubulin probe.

RESULTS

Effects of RA on Cell Growth. SSV-NRK cells (7.5×10^4) were grown on duplicate 60-mm Petri dishes in a series of dilutions of RA. The growth of these cells was inhibited by RA in a dose-dependent manner: 85% of controls at 10^{-7} M RA, 70% at 10^{-6} M RA, and 60% at 10^{-5} M RA. Morphological changes suggestive of flat reversion were also observed in all RA-treated cultures, as early as 24 h in 10^{-5} M RA. Our results agree with published data that anchorage-dependent growth of SSV-NRK cells was inhibited by RA at the median effective dose of 5×10^{-6} M (8).

Effects of RA on c-*fos* Expression. The expression of c-*fos* has been reported to be one of the earliest responses in fibroblasts after mitogenic stimulation (13). We assayed c-*fos* mRNA in response to serum stimulation in RA-treated cells. Cells were grown to confluence and serum starved by maintenance in 0.5% FBS for 48 h. During this time, they divided extremely slowly (probably in response to constitutively expressing p28^{sis}). RA at 10^{-5} M was then added to the cultures for 6 h before harvesting for RNA extraction. Mitogenic stimulation was achieved by incubating the cells in 20% FBS during the last 30 min of RA treatment. The Northern blots revealed a significant decrease in the steady-state level of c-*fos* mRNA in treated cells (Fig. 1*A*, *Lane 1*) relative to the untreated control cells (Fig. 1*A*, *Lane 2*). Densitometric measurements determined the amount of c-*fos* mRNA in RA-treated cells to be one-fourth that in the untreated controls, as normalized against tubulin bands. The experiment was repeated four times, giving an average 6-fold reduction of c-*fos* expression. Chronic (1 week) exposure of SSV-NRK cells to 10^{-6} RA resulted in a similar decrease in c-*fos* mRNA levels (data not shown). RA also blocked the induction of two other serum-inducible nuclear oncogenes, c-*jun* and, to a lesser extent, c-*myc* (data not shown). Our data (Fig. 1*B*) further confirmed that RA did not inhibit v-*sis* transcription in SSV-transformed fibroblasts, as previously reported (8).

Half-Life of c-*fos* mRNA in RA-treated Cells. Since RA may down-regulate the c-*fos* mRNA levels by increasing its degradation, we performed actinomycin D experiments to measure the rate of c-*fos* mRNA decay. SSV-NRK cells were grown to confluence, serum starved, and serum induced as described above. Immediately following a 30-min period of serum induction, actinomycin D at 30 μg/ml was added. The cells were harvested at the time of actinomycin D addition and at 15-min intervals thereafter. RA was added 6 h before actinomycin D and maintained in the cultures until harvesting. The decay of c-*fos* mRNA in the control was very rapid (Fig. 2), with a half-life of 12 min, which is in agreement with previous reports (24, 25). The half-life of c-*fos* mRNA in RA-treated cells was estimated to be 17.5 min. The low level expression of c-*fos* in the drug-treated cells makes the half-life estimate rather inaccurate. It is clear, however, that based on two independent experiments, the c-*fos* mRNA decay in RA-treated cells was not accelerated. Thus, RA does not appear to down-regulate the serum-induced c-*fos* mRNA level by increasing its turnover.

c-*fos* Transcription Initiation and Elongation. An alternative site of action of RA in down-regulating c-*fos* expression is at the level of transcription initiation and elongation. Therefore, we performed nuclear run-on experiments. SSV-NRK cells were grown to confluence in 10% FBS, serum starved for 48 h, and serum induced for 30 min with 20% FBS. Cells were grown in the presence of 10^{-5} M RA for 7 h before harvesting. Fig. 3 shows that the transcription of the first exon of c-*fos* in RA-treated cells was much reduced. From densitometric analyses, the intensity of the band corresponding to the first exon is decreased 4-fold in RA-treated cells as compared to control cells (after correcting for small differences in the 5S RNA-positive controls). The difference between RA-treated and con-

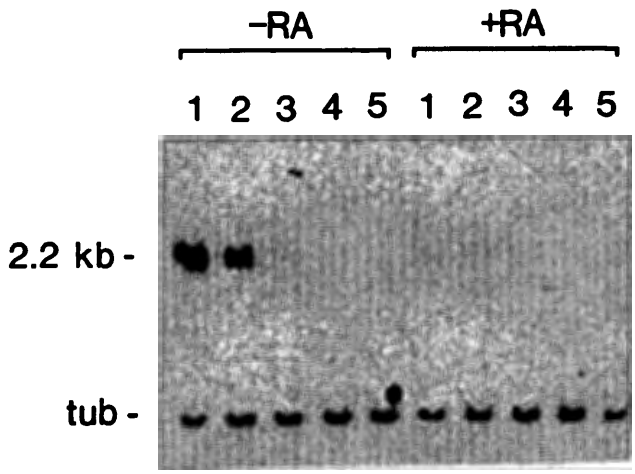

Fig. 2. Determination of the half-life of c-*fos* mRNA. Confluent, serum-starved cells were exposed to 10^{-5} M RA 5.5 h prior to serum stimulation. Twenty % FBS containing RA was then added and 30 min later 30 μg/ml actinomycin D was added. Cells were harvested at time 0, *i.e.*, the time of actinomycin addition (*Lane 1*), and at 15-min intervals thereafter (*Lanes 2, 3, 4,* and *5*). RNA was extracted and analyzed by Northern hybridization techniques. The 2.2-kilobase (*kb*) band is the c-*fos* mRNA. +RA, with RA treatment; −RA, without RA treatment. *tub*, tubulin.

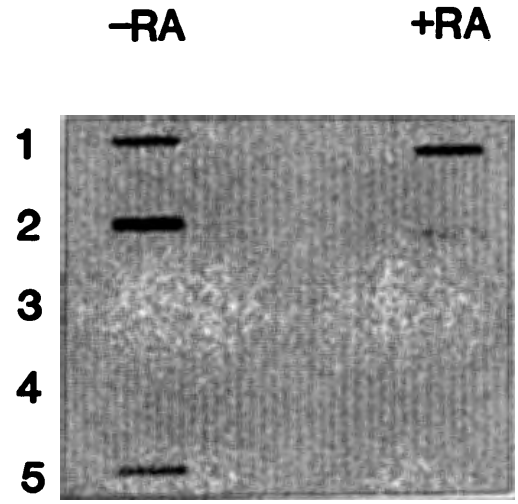

Fig. 3. Nuclear run-on analysis of c-*fos* transcription. SSV-NRK cells were serum starved and serum stimulated as described in text. RA (10^{-5} M) was added to the culture 6.5 h before serum stimulation and the cells were maintained in 20% FBS for 30 min until harvesting. The nuclear run-on experiments were performed as described in "Materials and Methods." DNA blotted on the membrane was for detecting: *Slot 1*, 5S RNA; *Slot 2*, the sense transcript of the c-*fos* gene exon 1; *Slot 3*, the antisense transcript of exon 1; *Slot 4*, the antisense transcript of exons 3 and 4; and *Slot 5*, the sense transcript of exons 3 and 4. +RA, with RA treatment; −RA, without RA treatment.

trol cells in the intensities of the bands corresponding to the further downstream third and fourth exons is also about 4-fold, indicating that transcription elongation does not appear to be affected by RA. We conclude therefore that the major effect of RA on c-*fos* mRNA transcription is its inhibition of transcription initiation.

It was noted here and by others (25) that transcription of c-*fos* mRNA is apparently attenuated. The ratio of signals for the first exon and to that for the third and fourth exons is 2.5 in untreated cells, after adjusting for slight differences in size of the probes. The attenuation is similarly observed in RA-treated cells, meaning that RA does not affect the rate of elongation.

Effect of Cycloheximide. Cycloheximide, an inhibitor of protein synthesis, has been shown to increase the level of c-*fos* mRNA in quiescent 3T3 cells and also their serum-induced level (13). Termed superinduction, this phenomenon has been explained on the basis of the inhibition by the drug of the synthesis of a labile nuclease that degrades mRNA or of the contact between the mRNA and a ribosome-associated nuclease (24). We tested the effect of RA on the cycloheximide-superinduced c-*fos* mRNA levels. SSV-NRK cells were starved and serum stimulated as before. Cycloheximide, 50 μg/ml, was added 3 h prior to serum stimulation. As shown in Fig. 4, cycloheximide treatment resulted in a 3-fold increase in the c-*fos* mRNA level (*Lane 3*). RA blocked c-*fos* induction by serum in the presence of cycloheximide (*Lane 4*), consistent with the notion that RA acts at the level of transcription initiation, whereas the effect of cycloheximide is at the level of mRNA turnover.

Does RA Regulate c-*fos* mRNA Level by Directly Interfering with a Membrane Signal Transduction Mechanism? The mechanism of action of RA at the molecular level is not fully understood. Earlier models for retinoid action proposed the involvement of both calcium-cyclic AMP and phosphatidyl inositol membrane signal transduction pathways (26–28). The induction of c-*fos* in fibroblasts following the stimulation by growth factor is very rapid, usually within 5 min, reaching a steady-state level in about 30 min (12, 13). To test whether RA interferes with the signal transduction pathway at the membrane level, we examined the response of c-*fos* expression to short-term RA treatment. SSV-NRK cells were grown in 10% FBS and starved in 0.5% FBS for 2 days. RA was added to the serum-starved cultures to a final concentration of 10^{-5} M 5 min before addition of 20% FBS. This would ensure that RA could take effect before the phosphatidyl inositol cascade would be activated by serum (in a matter of seconds) (12). The cells were then incubated for an additional 25 min before harvesting. The results demonstrated that a 30-min exposure to RA was insufficient to cause a decrease in c-*fos* mRNA levels in SSV-NRK cells (Fig. 5). A most likely explanation for this observation is that RA, a lipid-soluble substance, does not directly interfere with the initial phase of signal transduction pathway.

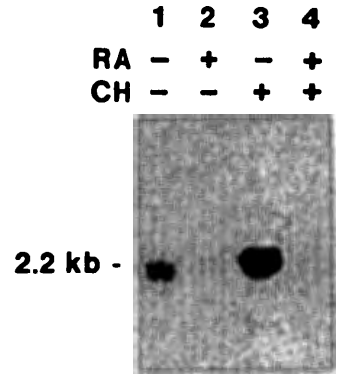

Fig. 4. Cycloheximide does not block the RA effect on c-*fos* induction. The SSV-NRK cells were starved and serum stimulated as before. RA and cycloheximide were added 6 and 3 h before serum stimulation, respectively; 2.2 kilobases (*kb*) indicates the size of c-*fos* mRNA. *CH*, cycloheximide.

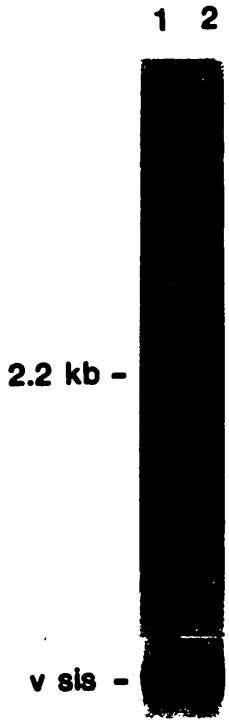

Fig. 5. Lack of effect on c-*fos* expression in cells treated with RA for only 30 min. The SSV-NRK cells were starved and serum stimulated as before. RA, 10^{-5} M, was added 5 min before serum stimulation. Cells were harvested 25 min after the addition of 20% fetal bovine serum containing RA for RNA extraction. *Lane 1*, with RA, and *Lane 2*, without RA treatment; 2.2 kilobases (*kb*) indicates the c-*fos* mRNA band. The same blot was stripped and rehybridized with the v-*sis* probe.

DISCUSSION

Data presented in this paper demonstrate that RA blocks the induction of c-*fos* mRNA in the simian sarcoma virus-transformed NRK fibroblast line. The inhibition occurs at the level of transcription initiation, as shown in the actinomycin experiments. The results of the cycloheximide experiments are consistent with this interpretation and further suggest that a labile protein factor is not required for the RA action. Our data also show that the RA effects are unlikely to be due to interference of the membrane events. Because c-*fos* induction is a critical event in transition of G_0-G_1 to S in quiescent fibroblasts upon serum stimulation (11), the inhibition of c-*fos* expression may account for its inhibition of the reentry of the serum-starved fibroblasts into cell division cycle.

c-*fos* is a nuclear oncogene, encoding a product which forms a transcription factor AP-1 with c-Jun (29, 30). AP-1 is known to mediate activation of transcription by serum. The c-*fos* gene promoter itself contains an AP-1-like sequence (31), suggesting that the gene is positively autoregulated. Other *cis*-acting elements of the gene include the adjoining SRE that binds to SRF (32, 33). The c-Fos protein, in addition, represses (negatively autoregulates) the c-*fos* promotor by interacting with the SRF, by an as yet uncharacterized mechanism (34, 35). This opposing effect of c-Fos protein appears paradoxical, but it may serve as a safeguard against excessive accumulation of c-*fos* mRNA in the cells following serum stimulation (34). A possible site of RA inhibition of c-*fos* transcription is a *cis*-acting element within or near SRE. Alternatively, RA may not directly contact DNA, but rather interacts indirectly with the c-*fos* regulatory elements through other protein factors.

RAR α, β, and γ belong to the steroid/thyroid hormone/retinoic acid receptor superfamily (36, 37). The receptors, upon binding to the ligands, recognize specific sequences that are localized upstream from a target gene, resulting in activation (or repression) of that gene. A RAR-binding sequence has been identified as a direct repeat of the motif GTTCAC (38). A simple explanation of our results is that the RAR binds to the retinoic acid response element near SRE. In support of this notion, Schüle *et al.* reported that AP-1 and RAR recognize a common response element in the human osteocalcin gene (39). However, a search for the RAR-binding sequence within approximately 280 base pairs 5′ and 3′ of SRE in the murine c-*fos* gene (available in GENBANK) has been unsuccessful. It is more likely then that RAR interacts with SRF or other *trans*-activating factors that regulate c-*fos* expression. This possibility was raised by recent reports that negative regulation of genes by the glucocorticoid hormone could occur via protein-protein interaction between c-Jun and the hormone receptor (40, 41).

In the HL-60 human promyelocytic cell line differentiating into granulocytes, c-*fos* expression has been shown to be up-regulated by RA (42), and this up-regulation appears to play a role in their differentiation. c-*fos* up-regulation is also reported for differentiating F9 murine teratocarcinoma cells (43). In a human embryonal carcinoma cell line, NT2/D1, RA has minimal effects on c-*fos* expression (44). To our knowledge, ours is the first case where c-*fos* expression is down-regulated by RA in a cell line. The finding may be important in explaining some of the antitumor effects of RA.

ACKNOWLEDGMENTS

We thank Dr. S. Aaronson for SSV-NRK cells; Dr. I. Verma for a c-*jun* plasmid, pcD10; Dr. E. A. Thompson for assistance in the nuclear run-on experiments and critical review of the manuscript; and Drs. T. Sato and I. Boldogh for advice.

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