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J A Schalken, … , H P Bloemers, W J Van de Ven

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Research Article

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fur Gene Expression as a Discriminating Marker for Small Cell and Nonsmall Cell Lung Carcinomas

Jack A. Schalken,* Anton J. M. Roebroek,‡ Paul P. C. A. Oomen,§ Sjoerd Sc. Wagenaar,|| Frans M. J. Debruyne,* Henri P. J. Bloemers,‡ and Wim J. M. Van de Ven‡

**Department of Urology, University of Nijmegen, Geert Grooteplein Zuid 16, 6500 HB Nijmegen, The Netherlands; ‡Molecular Oncology Section, Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands; §Department of Physiological Chemistry, State University of Utrecht, Utrecht, The Netherlands; and ||Department of Pathology, St. Antonius Hospital, Nieuwegein, The Netherlands*

Abstract

The recently discovered *fur* gene encodes a membrane-associated protein with a recognition function. To further characterize the gene, we studied its expression by Northern blot analysis using poly(A)-selected RNA from a variety of organs of African green monkey and rat. The *fur* gene appeared to be differentially expressed, relatively high levels of *fur* mRNA being present in specimens of liver and kidney, low levels in brain, spleen, and thymus, and very low levels in heart muscle, lung, and testis. mRNA levels in specimens of human lung tissue without neoplastic lesions were also very low. Similar analyses of primary human lung carcinomas of different histopathological types revealed a highly selective and strong elevation of *fur* expression in nonsmall cell lung carcinomas, but not in small cell lung carcinomas. These results indicate that *fur* expression can be used to discriminate between these two types of human lung cancer.

Introduction

Recently, we have described a new gene that we designated *fur* (1). The gene was discovered when the regions immediately upstream of the human and feline *fes/fps* proto-oncogenes were compared by Southern blot and heteroduplex analysis in an attempt to define more precisely the 5′ ends of these proto-oncogenes. The *fur* transcription unit appeared to be distributed over a DNA region of ~ 10 kbp and to code for an mRNA of 4.5 kb. The poly(A) addition site of the *fur* messenger was found in very close proximity of the *fes/fps* proto-oncogene (1).

Nucleotide sequence analysis of *fur*-related cDNA clones showed that the 3′ end of the *fur* transcript was characterized by a noncoding region of ~ 1.6 kb (1, 2). Furthermore, it appeared from analyses of a 3.1-kbp cDNA clone that *fur* contained an open reading frame of at least 1,500 bp. In the amino acid sequence deduced from it, a hydrophobic region was present at a position of ~ 50 amino acid residues from the carboxy terminus. This potential transmembrane domain was very similar to the ones found in some proteins encoded by class II genes of the MHC. Upstream of the transmembrane domain, a cysteine-rich region was present. Cysteine-rich regions appear widely distributed among receptor proteins and, in some cases, may be involved in ligand binding; they are found in the human insulin receptor (3), the human epidermal growth factor receptor (4), the glucocorticoid receptor (5), the oestrogen receptor (4, 6), and the LDL receptor (7). Significant homology, especially with respect to the topography of cysteine residues, was found between the cysteine-rich regions of human epidermal growth factor receptor, human insulin receptor, and furin, the putative translational product of *fur* (2). A transmembrane domain adjacent to a cysteine-rich region, as in furin, is more often observed in receptors (3–8). The characteristics described above make furin a likely candidate for a receptor for an as yet unknown ligand.

Because of the structural properties of furin, it was of interest to study the expression pattern of *fur* in normal and neoplastic tissue. In the present report, we describe the results of such a study in which we used poly(A)-selected RNA from specimens of a number of different organs of African green monkey and rat, as well as from normal lung tissue that was free of neoplastic lesions and from human lung carcinomas of different histopathological types.

Methods

Lung carcinomas. Lung carcinoma specimens from 49 patients were selected from the files of the Pathology Department of the St. Antonius Hospital, Nieuwegein, The Netherlands. Surgical specimens were presented fresh and sterile to the pathologist immediately after extirpation, and tumor tissue was quickly frozen in liquid nitrogen and stored at −70°C. Tumors were characterized by routine microscopical and histopathological techniques and classified according to the criteria of the World Health Organization (9) as small cell lung carcinoma (SCLC)[1] (9 cases) or nonsmall cell lung carcinoma (NSCLC) (40 cases). All SCLCs were examined by electron microscopy to confirm the presence of neuro endocrine granules. The NSCLCs could be classified as squamous cell carcinomas (19 cases) and adenocarcinomas (21 cases).

Specimens of normal tissue. Specimens of normal tissue were obtained from cat, rat, mouse, and monkey. Specimens were quickly frozen in liquid nitrogen immediately upon removal from the animal and stored at −70°C.

DNA probes and hybridization. DNA probes were isolated and labeled as described (10). To study *fur* expression, a 3.1-kbp *fur*-specific cDNA probe was used that was isolated from an oligo (dT)-primed

Address reprint requests to Dr. W. J. M. Van de Ven, Molecular Oncology Section, Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, 6525 EZ Nijmegen, The Netherlands.

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1. *Abbreviations used in this paper:* SCLC, small cell lung carcinoma; NSCLC, non-SCLC.

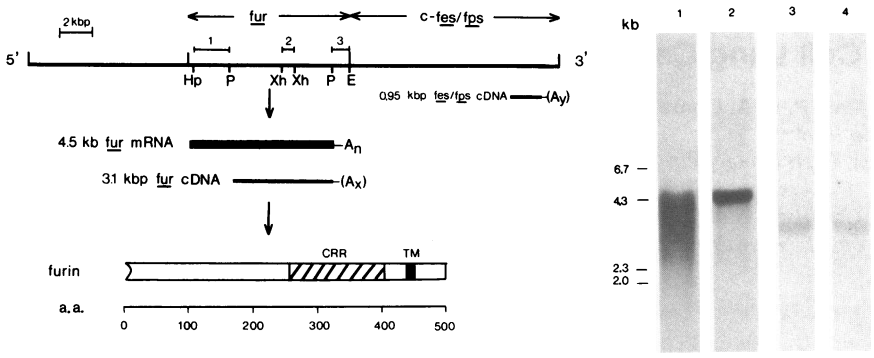

Figure 1. Schematic representation of characteristics of *fur*. At the top of the figure, the position of *fur* relative to the *fes/fps* proto-oncogene in the human genome is depicted. The three numbered bars represent *fur*-related genomic probes. The 4.5-kb *fur* mRNA is shown below the human genomic region followed by a 3.1-kb *fur* cDNA clone, which was used as a probe. At the bottom of the figure, the deduced *fur* translational product is represented as a bar. Under the bar representing the genomic DNA of the proto-oncogene a 0.95-kbp *fes/fps* cDNA clone (3) is shown. At the right of the figure, a Northern blot analysis of poly(A)-selected RNA from KG-1 cells is shown. Probes included a combination of genomic probes 1, 2, and 3 (lane *1*), the 3.1-kbp *fur* cDNA clone (lane *2*), the 0.95-kbp *fes/fps* cDNA clone (lane *3*), and the v-*fes*–specific S_1 probe (16) (lane *4*). Molecular weight markers include λ-DNA digested with restriction endonuclease Hind III. CRR, cysteine-rich region; TM, transmembrane domain; E, Eco RI; Hp, Hpa I; P, Pst I; Xh, Xho I.

cDNA library (2) from KG-1 cells (11). The 0.95-kbp *fes/fps*–specific cDNA probe was described before (1) and included the genetic sequences of the last five exons of c-*fes/fps* (12). To assay the presence of actin transcripts, pAct-1 a hybrid molecule of pBR322 and 1.2 kbp of actin-specific cDNA sequences of hamster origin (13) was used. Hybridization experiments on nitrocellulose membranes were performed as described previously (10). For hybridizations on nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL) the method of Church and Gilbert (14) was used. The nylon membranes were dehybridized by incubation in 5 mM Tris-HCl (pH 8.0), 2 mM EDTA and 0.1× Denhardt's solution (1× Denhardt's solution contains 0.02% (wt/vol) BSA, 0.02% (wt/vol) polyvinylpyrrolidone and 0.02% (wt/vol) ficoll at 65°C for 2 h. Each dehybridization was checked by autoradiography. Blots could be used several times without significant loss of signal.

mRNA isolation and Northern blot analysis. Total cellular RNA was isolated from normal and malignant lung tissue using the lithium–urea procedure described by Auffray and Rougeon (15). 10 μg of oligo (dT)-cellulose purified mRNA was glyoxalated and size fractionated on 1.0% agarose gels and transferred to Hybond-N (procedure as recommended by Amersham Corp.).

Results

Differential expression of fur in normal tissues of monkey and rat. We have studied the expression pattern of the recently discovered *fur* gene in normal tissues of different histological types by Northern blot analysis. For this study, a suitable *fur*-specific probe was selected. In Fig. 1, characteristics of the *fur* gene are summarized. With a combination of genomic probes representing a major portion of the *fur* transcription unit (probes 1, 2, and 3; see Fig. 1) the 4.5-kb *fur* transcript could be detected in poly(A)-selected RNA from the human cell line KG-1. However, a smear of transcripts of lower molecular weight was always observed (Fig. 1, lane *1*). When a 3.1-kbp *fur*-specific cDNA clone (Fig. 1) was used, a clear and distinct band was obtained instead (Fig. 1, lane *2*). As can be seen in Fig. 1, this cDNA clone represented about 70% of the 4.5-kbp *fur* transcript and contained the genetic sequences that encode the transmembrane domain and the cysteine-rich region. The smearing effect observed with the combined genomic probes was probably due to repetitive sequences most likely located in the 5′ region of *fur* (data not shown). To study the *fes/fps* transcripts, a 0.95-kbp *fes/fps*-specific cDNA was used (Fig. 1, lane *3*) instead of the v-*fes*–specific S_1 probe (16) (Fig. 1, lane *4*).

The facts that the *fur* cDNA probe was of human origin as well as that the availability of specimens from a number of different human organs was rather limited led us to test the evolutionary conservation of the *fur* gene to select an appropriate alternative. This was performed by Northern blot analysis of poly(A)-selected RNA from kidney specimens of mouse, monkey, man, rat, and cat (Fig. 2). Although some variation in the signal strength could be observed, the *fur* gene seemed fairly well conserved during evolution and could be detected in all these species under hybridization conditions of high stringency. The mRNA of rat appeared a little shorter as could be observed upon prolonged agarose gel electrophoresis (data not shown). Based on these observations, we chose to test *fur* expression in various tissues of African green monkey and rat. In an attempt to compare similar amounts of poly(A)-selected RNA, we used OD_{260} and ethidium bromide staining to estimate RNA concentrations. Results of Northern blot analysis of poly(A)-selected RNA from a number of tissues of African green monkey are summarized in Fig. 3. Relatively high levels of *fur* transcripts were found in RNA from liver and kidney. Lower levels of *fur* expression were observed in brain, spleen, and thymus, and very low levels in heart muscle, lung, and testis. Expression of *fur* was also found in some established cell lines (data not shown), for instance in KG-1 cells (Fig. 1, lanes

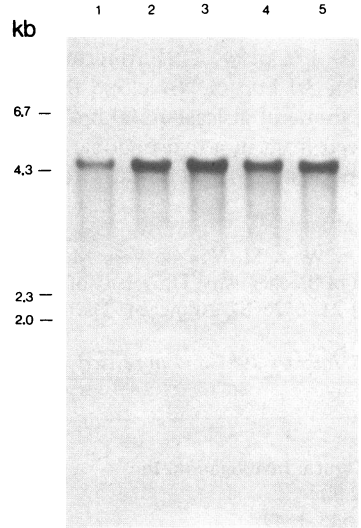

Figure 2. Northern blot analysis of poly(A)-selected RNA (20 μg amounts) isolated from kidney of mouse (lane *1*), monkey (lane *2*), man (lane *3*), rat (lane *4*), and cat (lane *5*). Molecular weight markers are the same as those described in the legend to Fig. 1.

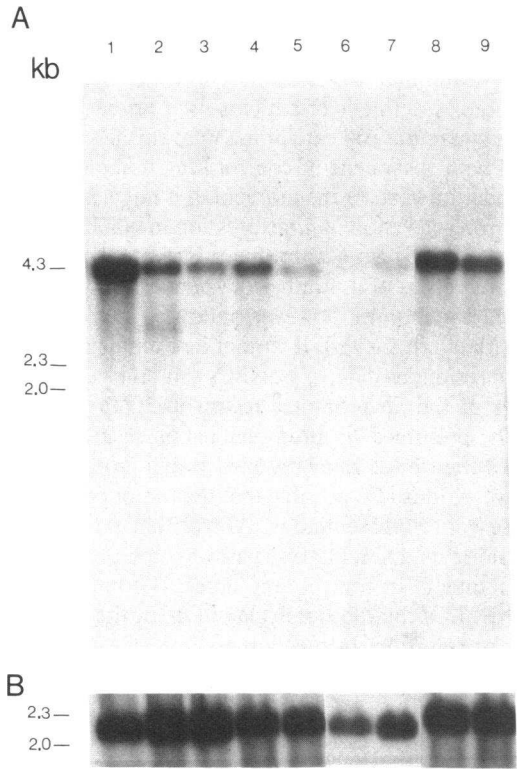

Figure 3. Expression of *fur* and *fes/fps* in a number of tissues of an African green monkey and Hela cells. (*A*) Northern blot analysis was performed with poly(A)-selected RNA (20 μg amounts) of liver (lane *1*), spleen (lane *2*), brain (lane *3*), thymus (lane *4*), lung (lane *5*), testis (lane *6*), heart muscle (lane *7*), kidney (lane *8*), and Hela cells (lane *9*). As a molecular probe, the 3.1-kbp *fur* cDNA was used. Upon autoradiography, the blot was screened with the 0.95-kb *fes/fps* cDNA. Molecular weight markers are the same as those described in the legend to Fig. 1. (*B*) The same blot as that used in part *A* of the figure was used in a Northern blot analysis with an actin-specific probe.

1 and *2*) and in Hela cells (Fig. 3, lane *9*). It should be emphasized that the autoradiograph shown in Fig. 3 *A* was exposed 24 h (using Kodak XAR-5 and two intensifying screens [Lightning Plus; DuPont Photo Products, Burbank, CA]). In Fig. 3 *B*, levels of actin transcripts in the various samples are shown (hybridization of the same Northern blot) as an additional control experiment. Exposure in this experiment was for 4 h. A similar expression pattern of *fur* was observed with RNA from various rat tissues (data not shown). These experiments indicate that the *fur* gene exhibits a differential expression pattern. At present, it is not clear whether the weak hybridization signals observed with specimens of some organs were due to low levels of *fur* transcripts in the tissue-specific cells of these specimens or to the presence of cells of hemopoietic origin that exhibit relatively higher levels of *fur* expression. In situ hybridization or immunofluorescence analysis could resolve this matter and establish in detail which cell types contain the *fur* transcripts or its translational product.

When the same blots were assayed for the presence of *fes/fps*–related sequences without prior dehybridization, a hybridization signal at 3.0 kb was observed only in spleen samples (Fig. 3 *A*, lane *2*). This observation is in agreement with previous reports indicating expression of *fes/fps* to be restricted mainly to hemopoietic cells of the myeloid lineage (17–21; see also Fig. 1, lanes *3* and *4*). Note that in Fig. 3 the *fur*-specific and the *fes/fps*–specific hybridization are superimposed, in which the *fur*-specific signal is the result of the first hybridization.

Expression of fur in human lung carcinomas. The observation that *fur* is expressed at very low levels in tissues like heart muscle, lung, and testis led us to extend our studies to naturally occurring tumors. Because of its high incidence in man, lung carcinomas were an obvious first choice for such a study.

We have studied nine cases of SCLC and 40 cases of NSCLC. The latter included 19 cases of squamous lung cell carcinoma and 21 cases of adenocarcinoma. For control experiments we used specimens of lung tissue without neoplastic lesions from man, cat, rat, and monkey. In Fig. 4 *A*, the results of a Northern blot analysis of similar amounts of poly(A)-selected RNA from the various types of lung carcinomas and the various control lung tissues are presented. Note that conditions were the same in all experiments and that exposure of the film was 10 h (using Kodak XAR-5 and two intensifying screens [Lightning Plus; DuPont Photo Products]). Normal lung tissue of man (lane *1*), cat (lane *2*), rat (lane *3*), and monkey (lane *4*) exhibited very low levels of *fur* expression (see also Fig. 3). Similarly, in SCLCs *fur* expression was also just above the detection level under these conditions. In the NSCLCs, however, strongly elevated levels of *fur* transcripts were observed (lanes *7–10*). We estimated these to be ~ 10 to 25 times higher than that in control lung tissue or SCLCs and ~ five- to tenfold higher than that in liver and kidney. No major differences in the levels of *fur* expression in squamous cell carcinomas and adenocarcinomas could be observed. In Fig. 4 *B*, the actin hybridization pattern of the same blot as

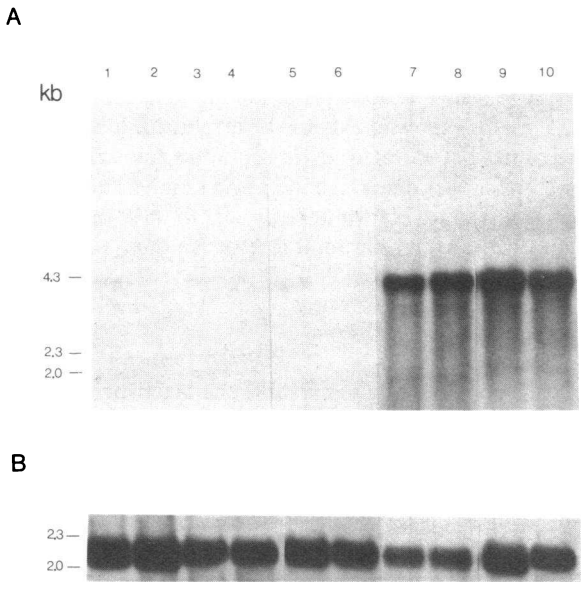

Figure 4. Expression of *fur* in normal and neoplastic lung tissue. (*A*) Northern blot analysis was performed with poly(A)-selected RNA (20 μg amounts) of normal lung tissue of man (lane *1*), cat (lane *2*), rat (lane *3*), and monkey (lane *4*), plus of two human SCLCs (lanes *5* and *6*), two human adenocarcinomas (lanes *7* and *8*), and two human squamous cell carcinomas (lanes *9* and *10*). As a molecular probe, the 3.1-kbp *fur* cDNA was used. (*B*) Control for the amounts of RNA on Hybond-N paper. The same blot as that used in part *A* of the figure was used in an hybridization analysis with an actin-specific probe.

Table I. Fur Gene Expression in Human Lung Carcinoma Specimens

Human lung tissue	Total numbers of specimens tested	Numbers in different categories of *fur* expression		
		++*	+‡	Control
SCLC	9	0	1	8
Squamous cell carcinoma	19	14	2	3
Adenocarcinoma	21	18	2	1
Control lung	5	—	—	5

* ++ Indicates a level of *fur* expression of 10 to 25 times higher than the level observed in control lung tissue specimens, as determined by densitometric measurements.
‡ + Indicates a level of *fur* expression of ~ five times higher than that observed in control lung tissue specimens, as determined by densitometric measurements.

used in part *A* is presented to indicate the relative amounts of actin mRNA on the blot. In Table I, the results of all lung carcinomas are summarized. In most NSCLC cases, levels of *fur* expression were 10 to 25 times higher than that in specimens of control lung tissue. In four cases, *fur* mRNA levels were ~ five times higher than control levels and in four other cases no elevation of *fur* transcription was observed. In the specimen of one of the nine SCLC patients, *fur* levels were elevated to some extent. The eight others were all very low.

The same Northern blots were also used to study expression of the *fes/fps* proto-oncogene. Expression of the proto-oncogene appeared to be low in all lung tissues tested, although some elevation of expression could be observed in some of the lung tumors tested (data not shown). The presence of low levels of *fes/fps* in lung tissue is already reported by others (17–21). However, it is not clear at the moment to which cell types the *fes/fps* expression should be attributed. It is possible that the somewhat elevated levels of *fes/fps* transcription in lung carcinomas was due to the increased numbers of alveolar macrophages that are known to be present in these tumors. In situ hybridization or immunofluorescence analysis should clarify this issue.

Discussion

The *fur* gene possesses two remarkable characteristics. First of all, its receptor-like structural features, the presence of a transmembrane domain and a cysteine-rich region, and, secondly, its evolutionary conserved location in the region immediately upstream of the *fes/fps* proto-oncogene. The fact that genetic sequences with coding potential for a protein with receptor-like characteristics remained closely linked to a proto-oncogene that encodes a tyrosine- specific protein kinase is interesting since it raises the possibility of a functional relationship. The expression pattern of the *fes/fps* proto-oncogene is known to be restricted to the myeloid lineage of hemopoietic cells (21). Analysis of the expression pattern of *fur* could, therefore, provide insight in a potential relationship between *fur* and the proto-oncogene. The differential expression pattern of *fur* described in this paper, however, shows differences in regulation of expression of *fur* and *fes/fps* implicating a functional role of the *fur* gene product that is not necessarily linked to the proto-oncogene product.

The observation that expression levels of *fur* transcripts in most lung tumor specimens of patients with primary adenocarcinoma or squamous cell carcinoma were strongly elevated as compared with specimens of control lung tissue or SCLC was of particular interest. At the moment, it is not clear how to explain the strong elevation of *fur* expression in NSCLCs. As a possible explanation, gene amplification was considered. Southern blot analysis of a number of adenocarcinomas and squamous cell carcinomas, however, indicated that this was not the case (data not shown). It cannot be excluded that the enhanced transcription of *fur* in NSCLCs should be explained by properties of the *fur*-promoter region itself. Preliminary analysis of the presumed *fur*-promoter region in a chloramphenicolacetyl transferase assay revealed strong promoter-like activity. However, it is also possible that the tumor cells in the NSCLCs arise from a differentiating cell type that expresses *fur* somewhere along its differentiating pathway and at the same time is low abundant in control lung tissue. Becoming abundant in the NSCLCs could be an explanation for the observed increase in *fur* expression. In such a model, the few cases that did not exhibit elevation of *fur* expression could also be explained.

Resected SCLC specimens are very uncommon, representing the minority of all cases. Thus, SCLCs amenable to surgical resection may be biologically different from SCLCs present in the standard unresectable manner. With respect to *fur* gene expression, we did not see such a biological difference in the nine SCLC cases we studied. Only one of them exhibited a slightly elevated level of *fur* transcripts. Of the nine SCLC specimens, four were obtained by surgical resection. Diagnosis of these four cases was based on histology, cytology, immunohistochemistry, and electron microscopy. Survival rates of the patients varied between 6 and 24 mo (average, 12.7 mo). Tumor specimens of the remaining five cases were obtained during autopsies that were performed within 1 h of the patient's death. Diagnosis of one of these cases was based on histology and immunohistochemistry (survival, 3 mo). Diagnosis of the remaining four cases was based on histology only. Survival rates of these four cases varied between 3 wk and 8 mo (average, 3 mo). In accordance with the diagnoses, all nine cases exhibited a biological behavior of small cell undifferentiated lung carcinoma.

The differential expression pattern of *fur* in SCLC and NSCLC also implied that *fur* gene expression could be used as a discriminating marker in studies on human lung cancer. At present, SCLC cells can be identified using a number of biomarkers (for a review see reference 22). For example, gastrin-releasing peptide is shown to be a suitable marker for SCLC cells (23). A similarly useful marker for NSCLCs, which account for ~ 75–80% of all cases of primary lung cancer, is not available at present (22). The identification of *fur* as a potential marker for NSCLCs could, therefore, be of importance for lung cancer diagnosis. Analysis of the *fur* gene has so far (1, 2) revealed promising characteristics of the gene and its product. First of all, evidence that furin represents a cell surface receptor is in favor of relatively easy accessibility of the protein. Moreover, the fact that in NSCLCs the levels of *fur* transcripts are selectively and strongly elevated may further facilitate furin detection. Both these characteristics could make the *fur*

gene a valuable object for the development of reagents to detect NSCLC in an early stage.

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