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TATA box-independent transcription of the human tissue plasminogen activator gene initiates within a sequence conserved in related genes

Beric R. Henderson[a] and Merilyn J. Sleigh[b]

[a]*Medical Oncology Unit, Department of Medicine, University of Sydney, Westmead Hospital, Westmead NSW 2145, Australia* and [b]*Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Biomedical Engineering, 103 Delhi Road, North Ryde, NSW 2113, Australia*

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Transcription of the human tissue-type plasminogen activator (tPA) gene has been reported to initiate from a single site proximal to a TATA box motif [1985, J. Biol. Chem. 260, 11223–11230]. In this study, we utilized primer extension analysis to evaluate the tPA mRNA start site in phorbol-12-myristate 13-acetate (PMA) induced WI-38 human lung fibroblast cells. Whilst some tPA mRNA initiated from the predicted TATA-proximal location (+1), a 10-fold greater proportion of tPA mRNA transcripts initiated 110 bases downstream from a sequence conserved and utilized as the TATA-independent transcription start site in the rodent tPA genes. Moreover, the transfection and expression in different cell types of a cosmid containing the entire human tPA gene resulted in utilization of the same downstream (+110) start site. We propose that this, rather than the previously published position, is the major transcriptional initiation point for the human tPA gene. A core sequence (5'-CAGAGCTG-3') was identified which is common to the TATA-independent mRNA start sites of the human, mouse and rat tPA genes, and which demonstrates only partial similarity to sequences found at the initiation point of other TATA-independent genes.

Tissue plasminogen activator; Transcription; TATA-box; mRNA

1. INTRODUCTION

Tissue-type plasminogen activator (tPA) is a serine protease which is capable of specifically converting the inactive zymogen plasminogen into plasmin, a broad substrate protease (reviewed in [1]). It is through the activation of plasmin that tPA is thought to play an important role in diverse cellular processes which include fibrin blood clot dissolution and cell migration [1,2]. The genes encoding human [3,4], mouse [5] and rat [6] tPA , and their promoters, have been isolated and studied. The human tPA gene promoter shares 55–60% sequence similarity with the rodent tPA promoters [6]. However, whilst transcription has been reported to initiate from a single site 25 bp downstream of a TATA box motif in the human tPA gene promoter [3,4], the rodent tPA promoters lack a functional TATA box at this position and initiates transcription from a conserved site approximately 110 bp downstream of the human tPA mRNA start site [5,6].

Abbreviations: bp, base pair; CAT, chloramphenicol acetyl transferase; cDNA, complementary DNA; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMA, phorbol-12-myristate-13-acetate; tPA, tissue-type plasminogen activator.

Correspondence address: B.R. Henderson, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges s/Lausanne, Switzerland. Fax: (41) (21) 652 6933.

Analysis of published gene sequences showed that the region that includes the transcription initiation site in the rodent tPA genes is also conserved in the human tPA promoter. However, the radiolabeled probes employed to map the human tPA mRNA start site [3,4], would have been of insufficient length to detect transcripts initiating from the site equivalent to that utilized in the mouse and rat tPA genes. In this communication we report that for the human tPA gene expressed in several mouse and human cell types, the majority of transcripts initiate in a downstream region equivalent to that utilised in rodent tPA genes, rather than at the published, TATA-dependent start site.

2. MATERIALS AND METHODS

2.1. *Cell lines and maintenance*

WI-38 human fetal lung fibroblast cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and cultured in Hams F12 medium (Flow Laboratories, Mclean, Virginia) containing 10% fetal calf serum (FCS; Gibco, Wisconsin). Cells of the F9 mouse embryonal carcinoma line [7] were obtained from Dr. A. Levine and grown in Dulbecco's modified Eagles medium (DME) containing 10% heat-inactivated FCS. F9 cells (and transfected derivatives) were maintained on plastic plates or flasks coated with 0.1% gelatin. Mouse L fibroblast cells were from Colombia University via Dr. K. Raphael (CSIRO, Prospect, NSW). They were cultured in DME with 5% FCS plus 5% calf serum. The human melanoma cell line MM170 was supplied by Dr. Hillary Warren (Woden Valley Hospital, Canberra, Australia) and grown in DME containing 10% FCS. MM170 cells secrete tPA enzyme but express very low basal

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levels of tPA mRNA (data not shown). The level of tPA enzyme activity secreted by the various cell lines was determined by fibrin gel zymography [8]. Human and mouse tPA enzyme activities were distinguished on the basis of size: the proteins migrate at 70 kDa and 79 kDa, respectively [8,9]. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2.

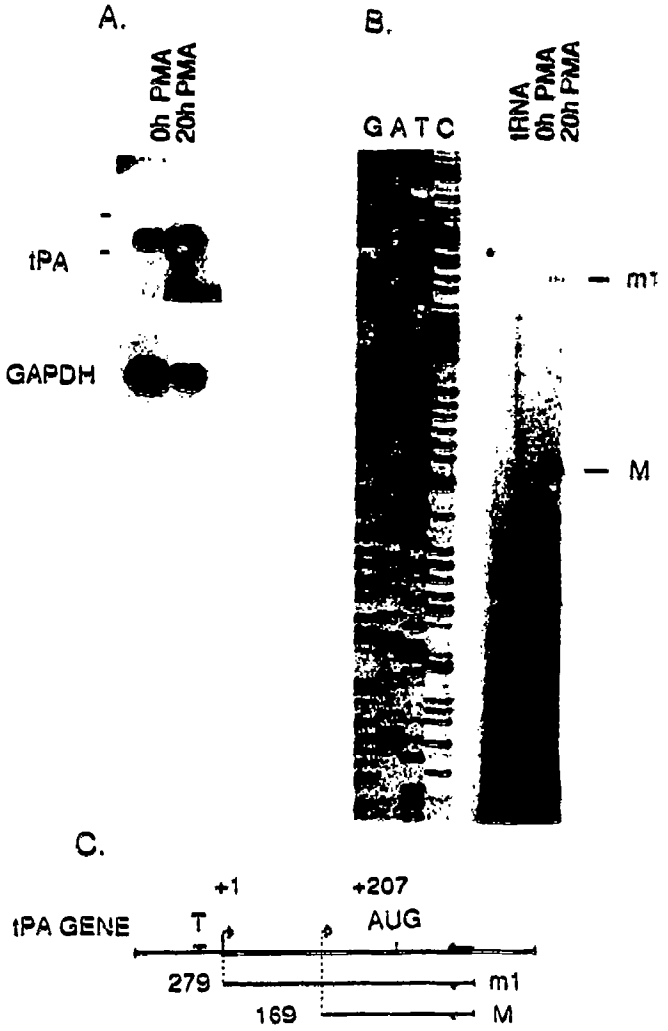

Fig. 1. Analysis of human tPA mRNA start sites. (A) Northern blot analysis of cytoplasmic RNA isolated from WI-38 cells, treated with 100 ng/ml PMA for 0 h or 20 h. 10 μg RNA was loaded per lane, the results shown representing non-adjacent lanes from the same autoradiograph. Filters were probed with ^{32}P-labeled human tPA (pPA 114B) and subsequently, GAPDH, cDNAs. Positions of 28S and 18S rRNA are indicated. (B) Primer extension analysis of tPA mRNA in PMA-induced WI-38 cells. 10 μg of tRNA, or cytoplasmic RNA from untreated or PMA-treated WI-38 cells, was annealed with radiolabeled primer (htPA-1) for 16 h, extended in the presence of AMV reverse transcriptase and size-separated on a 6% denaturing polyacrylamide gel. Dideoxy sequencing reactions of a standard insert were run in parallel for accurate size estimation (see below). The gel shown is typical of at least 3 independent experiments. (C) Schematic diagram of human tPA gene promoter, htPA-1 primer (horizontal arrow) and extension products corresponding to bands m1 and M. Positions of the TATA box (T), previously identified transcription start site (+1; reference [3]) and the translation start codon (AUG) at 207 are indicated.

2.2. *Plasmid DNA construction*

A DNA fragment containing 475 bp of the upstream region, the reported transcription start site and 120 bp of the 5' untranslated region of the human tPA gene, was excised from the plasmid pBG198 [3] following digestion with *Xho*II, blunt-ending with Klenow DNA polymerase I and further digestion with *Eco*RI. This tPA promoter fragment was sub-cloned into pUC9, and later cloned into pSV$_2$CAT [10] from which the SV40 promoter/enhancer had been removed by deletion of an *Nde*I–*Hin*dIII fragment, generating the plasmid named tPACAT. Standard molecular cloning techniques were used [11]. The cosmid H9, containing a 39 kbp insert with the entire human tPA gene together with 4.2 kbp 5' of the published transcription initiation point [9], was kindly provided by Dr. Marcy MacDonald (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, The Netherlands).

2.3. *DNA transfection*

Stably transformed cell lines were derived by co-transfecting cells with 10 μg DNA of the cosmid H9 or plasmid tPACAT supercoiled DNA, and 2 μg DNA of the plasmid pSV$_2$NEO (obtained from Dr. P. Berg, Stanford University Medical Center, CA). Transfection of cells was as described previously [12]. Colonies of cells expressing the *neo* gene were selected by resistance to G418 (Geneticin, Sigma Chemical Co., St Louis, USA; 400 μg/ml). Approximately 60–200 colonies were pooled to provide bulk transfected cultures. Transfected cell lines were shown by Southern blotting of genomic DNA [11] to contain an average of 50–100 copies of the transfected plasmids (data not shown).

2.4. *RNA isolation and Northern blot analysis*

Total cytoplasmic RNA was isolated from 10^7 cells by the Nonidet P40 lysis method described elsewhere [13]. Northern blot analysis of RNA was performed as previously described [14], and the filter reprobed with cDNA from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (plasmid pHcGAP obtained from ATCC, Rockville, MD) to control for integrity and loading of RNA. Washed filters were exposed to X-ray film at −70°C in the presence of intensifying screens for 7 days.

2.5. *Primer extension assay*

Two oligonucleotide probes were used for primer extension analysis. htPA-1 was complementary to bases +252 to +279 of exon 2 in the human tPA gene [4] and shares only 50% similarity to DNA sequence from the same position in the mouse tPA gene [5]. CAT-1 was complementary to bases +15 to +34 of the chloramphenicol acetyl transferase (CAT) gene coding sequence. These oligonucleotides (synthesized by Dr. G. Both, CSIRO Division of Biomolecular Engineering, NSW) were 5'-end labeled using [γ-^{32}P]ATP and T4 DNA polynucleotide kinase [11]. Labeled primer (1 ng) was hybridized with 10 μg of cytoplasmic RNA, in the presence of 40 mM NaCl, 10 mM Pipes pH 6.4, 2 mM dithiothreitol and 5 U RNA Guard (Amersham, UK) in 10 μl total volume, sealed in glass capillary tubes at 53°C for 16 h, as described elsewhere [15]. The annealed primer was then extended in the presence of 10 U AMV reverse transcriptase, 50 mM Tris-HCl, pH 8.3, 10 mM dithiothreitol, 6 mM $MgCl_2$ and 0.5 mM dNTPs, as described [15]. The extension products were precipitated in ethanol, denatured and subjected to electrophoresis on a 6% (w/v) polyacrylamide, 7 M urea gel, in parallel with dideoxy sequencing reactions [16] and/or size markers comprising ^{32}P-labeled *Hpa*II-digested pBR322 DNA.

3. RESULTS

3.1. *Identification of the tPA gene transcription initiation site in human cells*

Northern blot analysis revealed that tPA mRNA levels increased 8-fold following a 20 h treatment of WI-38

lung fibroblasts with 100 ng/ml PMA, as estimated by densitometry of the autoradiograph shown in Fig. 1A. This induction was specific as seen by comparison to a GAPDH reference gene (Fig. 1A). The tPA mRNA start sites were mapped in these cells by primer extension analysis (Fig. 1B). Two products resulted from primer extension of tPA transcripts in RNA from PMA-treated WI-38 cells. A minor band (m1) of 279 nucleotides corresponded to the previously described tPA mRNA start site [3,4] while the major product was approximately 169 nucleotides long (M) (Fig. 1B,C). No extension products were observed using tRNA as substrate (Fig. 1B), and bands m1 and M appear to be the only specific extension products, as they were the only bands confirmed by S1-nuclease mapping of the same RNA samples (data not shown). Band M was the only primer extension product observed following primer extension with RNA from human umbilical vein endothelial cells (data not shown) and untreated WI-38 cells (only just detectable following longer exposure of gel in Fig. 1B). The corresponding start site (M) is therefore utilized for basal transcription in at least two human cell types. The initiation site for transcripts generating the extension product M would map 110 bp downstream of the TATA-dependent start site (+1) in the human tPA gene, suggesting usage of the start site equivalent to that used in the rodent tPA genes [6].

3.2. *Mapping the start site of mRNA expressed from a transfected human tPA gene and promoter constructs*

The human tPA gene was transfected in cosmid form (H9) into mouse F9 cells which express little or no tPA [5], and mouse L cells which also do not express tPA [17]. Stable transfected cell lines were established, two of which (F9 PA124 and L PA111) expressed human tPA mRNA and enzyme activity (M.J. Sleigh, unpublished results). A low level of endogenous mouse tPA activity was detected in F9 PA124 cells (about 5% the level of human tPA expressed) but not in L PA111 cells (M.J. Sleigh, unpublished data). Primer extension mapping of tPA transcripts from these cell lines (Fig. 2A) revealed the same products M and m1 (detectable only upon longer exposures) as were observed in PMA-induced WI-38 cells. The m1 product was less than 2% of the total in the mouse cell lines. Two additional bands of 142 nucleotides (m3) and 196 nucleotides (m2) were consistently observed only in the transfected F9 cell line (Fig. 2A, and data not shown). Start site m2 lies 27 bases upstream of the conserved start site M, at a position also used as a mRNA start site by the endogenous mouse tPA gene [5]. The downstream m3 start site sequence is not conserved in the rodent tPA genes. However it shares 7 out of 9 nucleotides with the sequence containing the major inititiation site of the TATA-deficient thymidylate synthase promoter [18]. No specific extension products were detected using RNA isolated

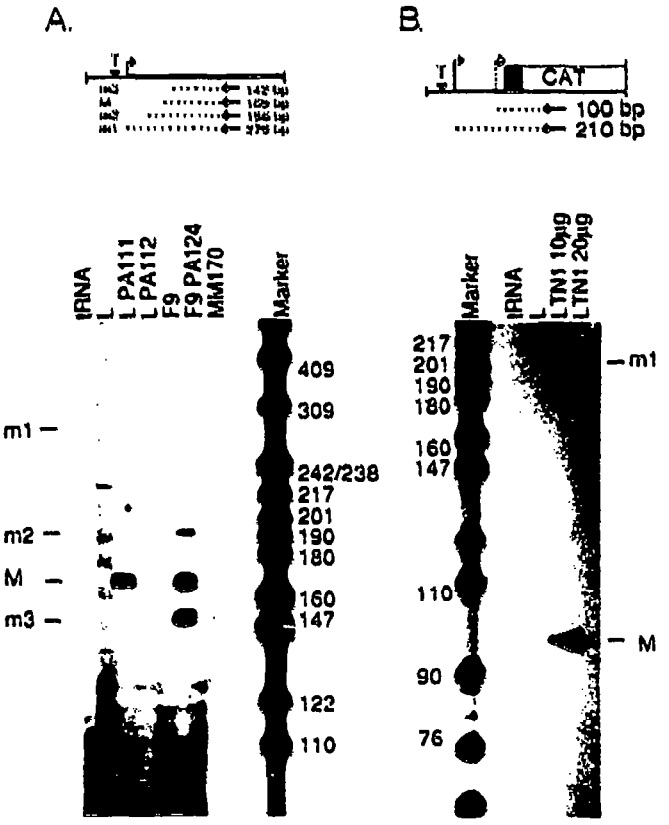

Fig. 2. Primer extension analysis of tPA mRNA start sites. (A) Total cytoplasmic RNA was isolated from mouse L cells or F9 cells, derivatives stably transfected with the human tPA gene cosmid H9 (L PA111, L PA112 and F9 PA124), and untransfected human MM170 cells. 10 µg tRNA or cytoplasmic RNA was hybridized with ^{32}P-labeled htPA-1 for 16 h and analysed by primer extension. Size standards are *Hpa*II-digested pBR322. Only the specific and reproducible primer extension products are indicated (m1, m2, M and m3), with their sizes. The TATA box (T), the previously proposed mRNA start site which corresponds to m1 (+1) (arrow) and the previously proposed transcribed sequence (thick line) are indicated. Although not so in this figure, band m3 was generally of equal signal intensity to that observed for band m2. (B) Primer extension using cytoplasmic RNA isolated from L cells, untransfected or stably-transfected with plasmid tPACAT (LTN1). 10 µg (or 20 µg LTN1 RNA as indicated) was hybridized with a ^{32}P-labeled CAT gene primer (+15 to +34) and analyzed as described in Section 2. The marker lane is *Hpa*II-digested pBR322 , with sizes shown in base pairs. Extension products are shown (m1, M) and sizes indicated on the schematic diagram at the top, wherein symbols are as described for Fig. 1.

from a line of transfected L cells expressing only very low levels of tPA (L PA112), non-transfected mouse L or F9 cells, or from human MM170 cells. Sizes of primer extension bands were estimated from restriction fragment markers and from parallel sequencing reactions (data not shown).

The position of transcription initiation in certain genes may be dependent on sequences downstream of the mRNA start site [19]. A plasmid tPACAT was therefore constructed. This plasmid contains 475 bp of the human tPA gene sequence including the proposed

major transcription initiation site (M) and only 8 nucleotides 3′ of this site. tPACAT was transfected into L cells and a stably-transfected, CAT mRNA-expressing cell line (LTN1) was established (B.R. Henderson, PhD thesis, University of Sydney, 1990). Primer extension (see Fig. 2B) using a CAT gene specific primer (see Section 2) detected one major band of 100 nucleotides in length corresponding in size to an RNA product initiating at the major start site M. A very faint 210 nucleotide band equivalent in length to RNA initiating from site m1 was also observed (data not shown). Extension products were undetectable using RNA isolated from non-transfected L cells.

4. DISCUSSION

The human tPA gene sequences described by Fisher et al. [3] and Friezner Degen et al. [4] revealed a TATA box 25 bp upstream from a site which they demonstrated by primer extension and S1-nuclease analysis to be a start site for gene transcription in human cells. The results described in this study however, demonstrate that in at least some situations, the major site for transcription initiation lies 110 base pairs downstream. The location of this major start site precluded its detection in the previous studies [3,4], as radiolabeled probes were employed which did not extend sufficiently downstream to enable its detection. This site is used to initiate transcription in normal human cells expressing the endogenous tPA gene, and in human cells induced to express higher levels of tPA by treatment with the tumor promoter phorbol-12-myristate-13-acetate.

The same site predominates when either the complete human tPA gene, or the promoter region attached to the CAT marker gene, are introduced into mouse cells (summarized in Fig. 3A). This indicates that utilization of the start site M may occur independently of species or state of induction of the cells, at least in those cell-lines described here.

The major transcription start site for the human tPA gene now described appears to be identical to that used in the rat and mouse tPA genes [5,6]. Alignment of human, rat and mouse tPA gene upstream sequences suggests a possible insertion in the human gene 5′-flanking region of a 31–36 nucleotide sequence which includes the TATA-box and the TATA-proximal start site, with no equivalent sequences detected in the other two genes (see [6] for sequence comparison). On the other hand, an 8 nucleotide sequence (5′-CAGAGCTG-3′) around the published initiation sites for the mouse and rat gene, and the major site now proposed for the human tPA gene, is highly conserved. It is possible that this conserved sequence element (shown in Fig. 3B) is sufficient to position and initiate tPA gene transcription efficiently. This postulate is indirectly supported by the observations that neither sequences downstream (tPACAT data, this study) nor upstream (mouse tPA

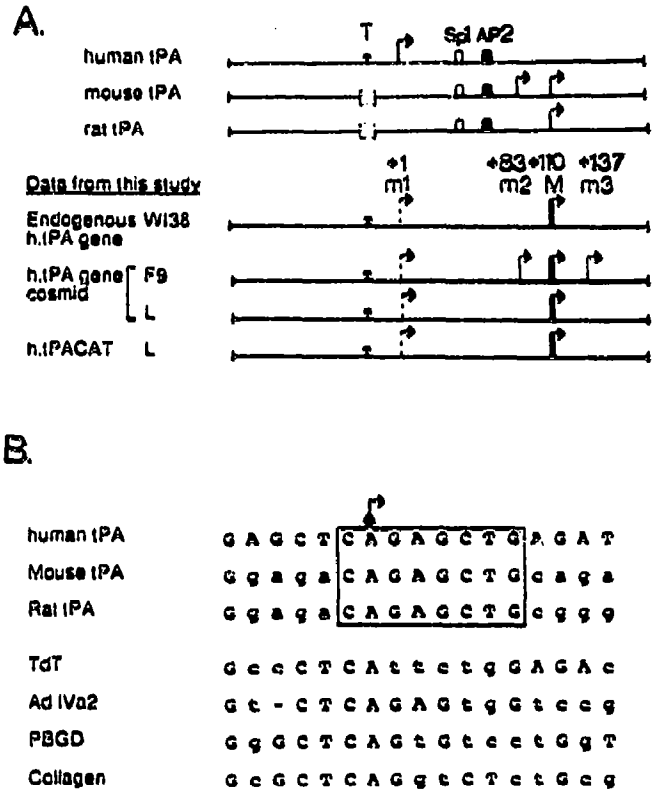

Fig. 3. Summary of human tPA mRNA start sites. (A) Diagram comparing the promoters and locations of published transcription start sites for human [3,4], mouse [5] and rat [6] tPA genes. The TATA box (T), initiation sites (arrows), and proposed Sp1 and AP2 binding sites are indicated. Open boxes show the positions of sequences absent in the mouse and rat tPA genes compared with the human tPA gene. Relative positions of the mRNA initiation sites (m1, m2, m3, M) determined by primer extension for the human tPA (h.tPA) gene in this study, are summarized for the different untransfected or transfected cell lines as indicated. (B) Comparison of the major human tPA mRNA initiation site and flanking sequence (−6 to +11) with the mRNA initiation sites in the TATA-less mouse and rat tPA genes, and other TATA-independent genes. The examples shown are for the terminal deoxynucleotidyl transferase (TdT) gene [20], Adenovirus IVa2 promoter [21], human porphobilinogen deaminase (PBGD) gene [22] and human collagen IV α1 promoter [23]. Transcription initiates at the indicated A residue in all promoters. A core sequence common to the tPA gene initiation sequences is boxed.

promoter analysis [5]) of this core sequence appear to be required for correct initiation to occur.

The sequence immediately flanking the M start site for the human tPA gene (−3 to +2) shows homology to the transcription initiation regions of other TATA-independent genes (sequences compared in Fig. 3B). However, only limited similarity exists between the conserved tPA initiation sequence 5′-CAGAGCTG-3′ and the initiator sequence (Inr) previously described for the TdT gene [20]. The recent observation that a DNA sequence corresponding to the conserved tPA initiator interacts specifically with nuclear proteins [24], further suggests that this sequence may represent an important

determinant of both basal and PMA-inducible tPA gene transcription initiation.

The data presented here provide evidence which suggests that the tPA genes may be transcriptionally regulated more similarly than was previously thought. This could prove important to our understanding of human tPA gene regulation/expression given that most studies on transcriptional induction of the tPA gene during ovulation and embryonic development have focussed on the rat (see [24]) and mouse (see [5]) tPA genes, respectively. While the downstream (M) site appears from our studies to be the predominant initiation point for transcription of the human tPA gene, it is possible that alternative sites, such as the TATA-proximal site m1, and also the m2 and m3 start points seen in cell line F9 PA124, are used preferentially in certain cell types and/or contribute to regulation of the human tPA gene. Further studies using appropriate extension primers will be needed to determine whether transcription initiation from site M predominates in all situations where the human tPA gene is expressed.

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