specific to this system. However, as I cannot expect that both the predator and the prey attain the highest efficiency in their interaction nor that any action of an agent affects the reproduction without a time lag, I speculate that the oscillation is not unique but rather a common process in polymorphic populations under natural conditions.

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Distinct Functions of SR Proteins in Alternative Pre-mRNA Splicing

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Alternative splicing of precursor messenger RNAs (pre-mRNAs) is a common mechanism of regulating gene expression. SR proteins are a family of pre-mRNA splicing factors that are structurally related and evolutionarily conserved. Any member of the SR family can complement a splicing-deficient extract that lacks the entire family of SR proteins. Here it is demonstrated that particular SR proteins have distinct functions in alternative pre-mRNA splicing in vitro. In addition, SR proteins are differentially expressed in a variety of tissues. These results suggest a fundamental role for SR proteins in the regulation of alternative splicing.

SR proteins comprise a family of evolutionarily conserved pre-mRNA splicing factors (*1*). The primary amino acid sequences of these proteins are highly related, and they share an NH_2-terminal RNA recognition motif (*2*) and a COOH-terminal domain of variable length that consists almost exclusively of alternating Ser and Arg residues (*1*, *3*). Many animal cells express a set of SR proteins of similar molecular mass: 20, 30, 40, 55, and 70 to 75 kD (*1*, *3*). The 30-kD band contains two distinct polypeptides, SRp30a and SRp30b, which have also been described as SF2/ASF (*4*, *5*) and PR264/SC35 (*6*), respectively. Furthermore, the sequences of individual SR proteins are highly conserved between species; for example, there is a 58% amino acid identity between nematode and human SRp30a in the non–Ser-Arg region (*7*).

Individual SR proteins isolated from vertebrates and invertebrates function similarly as essential pre-mRNA splicing factors when tested in depletion and reconstitution assays (*1*, *4*, *5*, *8*, *9*). These tests were possible because SR proteins are absent from a splicing-deficient extract (S100) of HeLa cells that is prepared by a 100,000*g* centrifugation of lysate containing 4.5 mM $MgCl_2$ (*10*). Under these conditions, SR proteins are insoluble (*1*, *3*). Addition of any one of four different SR proteins purified from calf thymus (*1*) or any one of five SR proteins purified from human HeLa cells (*11*) can complement this splicing-deficient extract so that in vitro splicing of pre-mRNAs containing single 5′ and 3′ splice junctions can then occur. It has also been shown that increasing the concentration of SRp30a (*4*, *5*) or SRp55 (*8*) results in a shift in the 5′ splice site that is used in the case of several pre-mRNAs that contain multiple 5′ splice sites. Although all SR proteins exhibit similar activity in these simple biochemical assays, the strict size and sequence conservation between vertebrates and invertebrates of the different SR proteins suggests that each SR protein has a distinct and essential function. Here, we present evidence that individual SR proteins allow the preferential use of different pre-mRNA 5′ splice sites.

As a first step toward a quantitative analysis of SR protein function in alternative pre-mRNA splicing in vitro, we determined the relative activity of several different SR proteins in splicing a single intron. SRp30b, SRp40, SRp55, and SRp70 were purified from calf thymus (Fig. 1), and we tested the proteins for their abilities to splice a β-globin pre-mRNA substrate (*12*) when they were added to the HeLa S100 splicing-deficient extract. The amount of each SR protein added was adjusted such that equivalent amounts of mature mRNA product were generated in each reaction (Fig. 2A). Quantitation of these reactions with the use of a phosphor imager (*13*) is shown (Fig. 3A). When increasing amounts of SR protein were added to the splicing-deficient extract, the amount of mRNA produced increased. This production was dose-dependent; that is, for each amount of any of the four SR proteins added, the amount of mRNA product appeared to be approximately equal. Splicing approached a maximal rate of 50% conversion to spliced products with the addition of 600 ng of each SR protein preparation. These results show that SR proteins function in a parallel and concentration-dependent manner (Figs. 2A and 3A). The concentration of each SR protein was found to be approximately 150 ng/μl (Fig. 1), and therefore the specific activity of each SR protein preparation was nearly identical.

To test the possibility that specific SR proteins confer preferential utilization of different splice sites, we used equivalent amounts of SRp30b, SRp40, SRp55, and SRp70 to splice other pre-mRNAs in a HeLa cell S100 splicing-deficient extract.

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SR proteins were first assayed for alternative 5′ splice site choices on an adenovirus pre-mRNA construct containing part of the *E1a* gene. Transcripts from the *E1a* gene are alternatively spliced at two different 5′ splice sites and at a unique 3′ splice site to yield 12S or 13S mRNA (*14*). The products of SR protein-dependent in vitro splicing with the *E1a* pre-mRNA construct are shown in Fig. 2B and quantitated in Fig. 3B. All of the SR proteins produce large amounts of the 13S splice product. However, whereas SRp30b promoted the use of the 13S splice site nearly exclusively, SRp40, SRp55, and SRp70 also promoted the use of the distal splice site and produced the 12S product RNA. Limiting concentrations of SRp40, SRp55, and SRp70 demonstrated no preference for either splice site, which contrasts with the strong preference of SRp30b at all concentrations for the proximal 13S splice site.

We also tested a simian virus 40 (SV40) pre-mRNA construct containing the t/T antigen intron and short regions of the flanking exons (*15*). This substrate pre-mRNA has two alternative 5′ splice sites and a fixed 3′ splice site. Proximal and distal 5′ splice sites are used to encode small t and large T antigens, respectively. The products of in vitro splicing with SR proteins on the alternative splicing of SV40 t/T antigen pre-mRNA. A control in vitro splicing reaction containing HeLa cell S100 extract, ^{32}P-labeled pSVi66 pre-mRNA splicing substrate, and 4 μl of buffer D without SR proteins is shown (lane 1). The products from identical reactions that contained 4 μl of SRp30b (lane 2) and 3 μl of SRp70 (lane 3) are shown. The products from reactions containing 4 μl of SRp30b and 3 μl of SRp70 (lane 4) are shown.

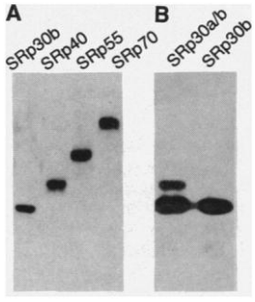

Fig. 1. SR protein purification. (**A**) Preparations of SRp30b, SRp40, SRp55, and SRp70 (5 μl) normalized for splicing of the β-globin intron were separated by SDS-PAGE and stained with Coomassie brilliant blue R. Comparison with bovine serum albumin standards indicated that the concentration of each SR protein was approximately 150 ng/μl. SR proteins were isolated from 600 g of calf thymus as described (*1*). SRp40, SRp55, and SRp70 were separated on several 10% polyacrylamide gels, excised, and eluted as described (*20*). The 30-kD SR protein band, which contains SRp30a and SRp30b, was also excised; SRp30b was then separated from SRp30a by a second round of SDS-PAGE separation on a 13.3% acrylamide and 0.67% bis-acrylamide gel. An immunoblot of the starting mixture of the two 30-kD SR proteins and the resulting purified SRp30b reacted with mAb104 (*3, 18*) is shown in (**B**). To confirm that this 30-kD SR protein was SRp30b, we sequenced two peptides from the pure protein (*21*) and found that both were identical to human SRp30b [DAEDAMDAMDGAVLDGR and VGDVYIPR (*22*)]. The eluted SR proteins were precipitated in 50% acetone at −20°C for 20 min and centrifuged in a microcentrifuge (Eppendorf) at −20°C for 15 min. Sedimented SR proteins were resuspended in 100 μl of 6.0 M guanidine HCl, dialyzed against buffer D containing 5% glycerol (*10*), reprecipitated with $MgCl_2$, and resuspended in buffer D containing 5% glycerol.

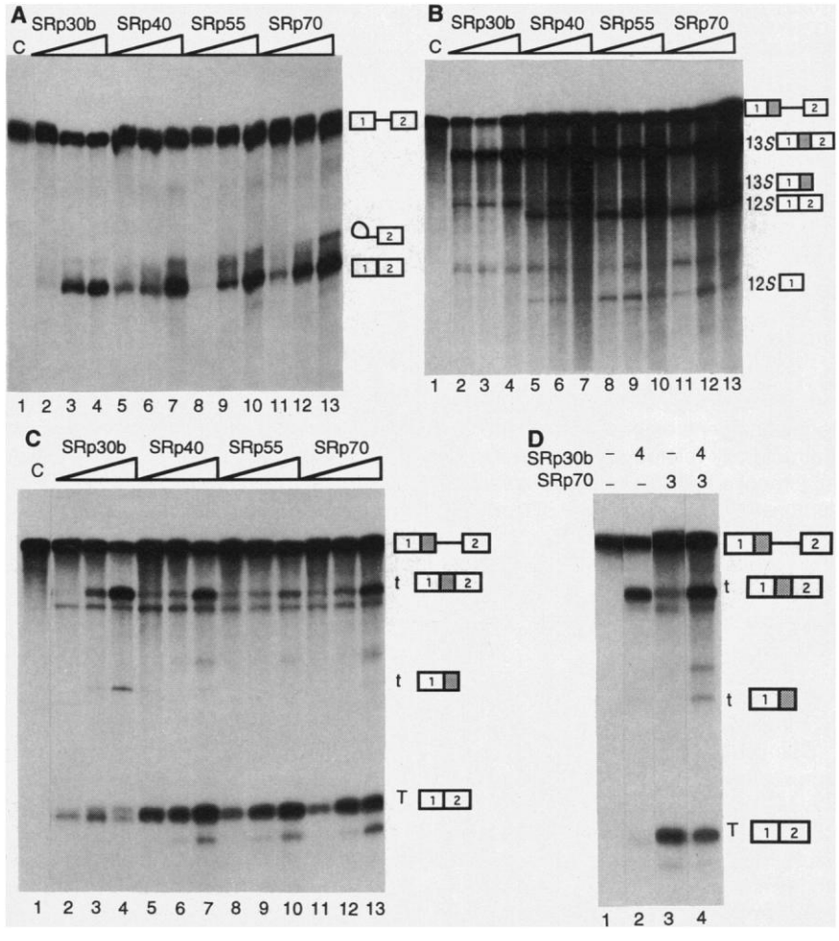

Fig. 2. Alternative splicing of pre-mRNAs containing multiple 5′ splice sites by different SR proteins. (**A**) Complementation of HeLa cell S100 splicing-deficient extract (*9, 10*) with SR proteins and a β-globin pre-mRNA construct. Lane 1 shows a control (C) in vitro splicing reaction containing HeLa cell S100 extract, ^{32}P-labeled SP64HβΔ6 (*12*) pre-mRNA splicing substrate, and 4 μl of buffer D (*10*) without SR proteins. Products from identical reactions that contained 1, 2, or 4 μl of SRp30b (lanes 2 through 4), SRp40 (lanes 5 through 7), SRp55 (lanes 8 through 10), or SRp70 (lanes 11 through 13) are shown. These are the same preparations of SR proteins as used in Fig. 1. Spliced products are indicated with exons shown as boxes, introns as lines, and intron lariats as loops. Preparation of HeLa S100 splicing extracts, preparation of ^{32}P-labeled substrate pre-mRNA, and in vitro splicing conditions were as described (*1*). In each case, the products were run on a 5% acrylamide (0.25% bis-acrylamide) denaturing gel and visualized by autoradiography. (**B**) Alternative splicing of adenovirus 2 *E1a* pre-mRNA in the presence of different SR proteins. We used the polymerase chain reaction to amplify a part of the *E1a* gene from adenovirus 2 genomic DNA. We then cloned this genomic fragment into pSP64 (Promega) and used it to synthesize substrate pre-mRNA corresponding to an *E1a* pre-mRNA that has been used before (*14*). Lane 1 shows a control (C) in vitro splicing reaction containing HeLa cell S100 extract and ^{32}P-labeled *E1a* pre-mRNA substrate. Products from identical reactions that contained 1, 2, or 4 μl of SRp30b (lanes 2 through 4), SRp40 (lanes 5 through 7), SRp55 (lanes 8 through 10), or SRp70 (lanes 11 through 13) are shown. Spliced products are indicated as in (A) with alternatively spliced exons shown as shaded boxes. (**C**) Alternative splicing of an SV40 t/T antigen pre-mRNA construct in the presence of different SR proteins. A control (C) in vitro splicing reaction containing HeLa cell S100 extract, ^{32}P-labeled pSVi66 (*15*) pre-mRNA splicing substrate, and 4 μl of buffer D without SR proteins is shown (lane 1). Products from identical reactions that contained 1, 2, or 4 μl of SRp30b (lanes 2 through 4), SRp40 (lanes 5 through 7), SRp55 (lanes 8 through 10), or SRp70 (lanes 11 through 13) are shown. Large T and small t antigen pre-mRNA splicing products are indicated. (**D**) Independent action of SR

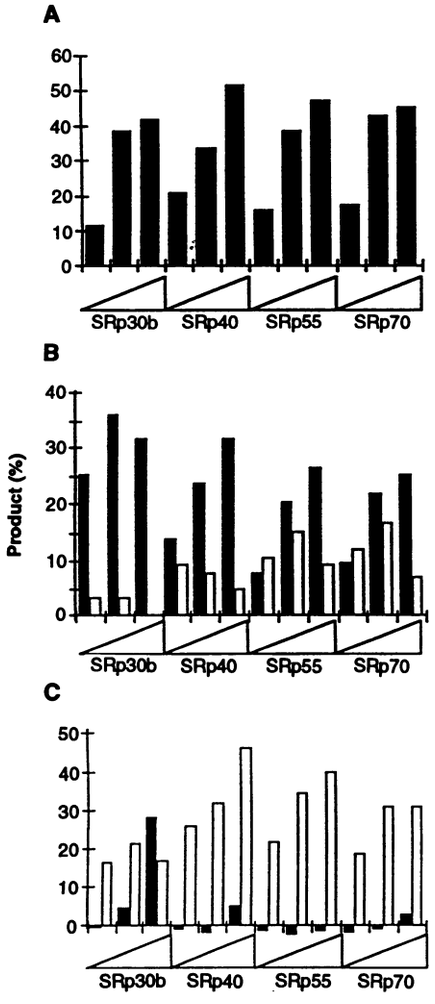

Fig. 3. Quantitation of alternative splicing with SR proteins. Quantitation of splicing reactions shown in Fig. 2, A through C, with a phosphor imager (*13*). To determine the percentage of each spliced mRNA produced in each reaction, we adjusted the values for counts per minute present in each band to normalize for the fact that larger RNAs contain more radioactivity. This was done by multiplying the counts per minute in each band by the ratio of the length of the pre-mRNA to the length of the spliced product. Then we divided the size-normalized counts per minute present in each spliced mRNA product band by the total of the size-normalized counts per minute present in the pre-mRNA and mRNA product bands for each lane. Background, determined by similar measurements taken from the nonsplicing control lane, was subtracted from each, and the values are shown in the bar graphs as percentages. (**A**) Quantitation of splicing of β-globin pre-mRNA with four individual SR proteins. (**B**) Quantitation of 13*S* and 12*S* spliced products in adenovirus with four individual SR proteins. Filled bars represent 13*S* mRNA, and open bars represent 12*S* mRNA. (**C**) Quantitation of large T and small t mRNA spliced products with four individual SR proteins. Filled bars represent small t antigen mRNA, and open bars represent large T antigen mRNA.

teins are shown (Fig. 2C), and the quantitation of these results is presented (Fig. 3C). SRp30b had a different effect in these reactions from that of SRp40, SRp55, and SRp70. Reactions containing SRp30b showed a slight preference for the large T antigen splice site at lower concentrations but a preference for the small t splice site at the highest concentration tested. In contrast, reactions with SRp40, SRp55, and SRp70 showed a strong preference for the large T antigen splice site at all concentrations. Minimal amounts of small t splicing were detected at the highest concentration each of SRp40 and SRp70. Addition of the largest amount (600 ng) of SRp30b (Fig. 2C, lane 4) and SRp70 (Fig. 2C, lane 13) resulted in ratios of the small t spliced product to the large T spliced product of 1.67 and 0.098, respectively. This is a 17-fold difference between SRp30 and SRp70 in the use of the two 5′ splice sites. When a combination of SRp30b plus SRp70 was assayed in this system, neither site predominated; that is, the ratio of small t to large T produced with SRp30b alone was 1.58 (Fig. 2D, lane 2) and with SRp70 alone was 0.023 (lane 3) but was 0.71 (lane 4) when the two were combined. The finding that the combined ratio was similar to the average of the ratios for two proteins assayed separately indicates that the two SR proteins act independently on individual pre-mRNA substrates to define the 5′ splice site.

The ability of SR proteins to direct the use of alternative 5′ splice sites implies that their distinct effects are the result of the differences in their primary amino acid sequences. We found that SR proteins subjected to guanidine renaturation complemented the S100 extract to splice the SV40 pre-mRNA with kinetics indistinguishable from those of untreated SR proteins, which indicates that this step of the protein purification was unlikely to introduce differences in the activities of different SR proteins (*16*). SRp40 and SRp55 function similarly, preferring both the SV40 large T 5′ splice site and either the adenovirus 12S or 13S 5′ splice sites. These two SR proteins contain an internal repeat domain related to the RNA recognition motif (*2*) shared by all SR proteins (*1*). In contrast, SRp30b preferred the SV40 small t and the adenovirus 13S 5′ splice sites. SRp30b does not contain the internal repeat of the RNA recognition motif. This correlation between the presence of the internal repeat and 5′ splice site selection suggests a specific role for this domain; this domain may in fact bind distinct RNA sequences involved in pre-mRNA splicing.

Many pre-mRNAs are alternatively spliced in a tissue-specific manner (*17*). If the relative amounts of SR proteins are important in the determination of splice site, then unique tissue-specific patterns of SR protein expression may contribute to the regulation of splice site selection. To pursue this possibility, we analyzed SR proteins prepared from calf tissues with the use of a monoclonal antibody (mAb104) that recognizes a conserved epitope on all SR proteins (*3*, *18*). Figure 4 shows an mAb104 immunoblot of equal amounts of total protein extracted from seven different calf tissues. Reproducible, tissue-specific differences both in the observed amounts of all SR proteins as well as in the relative amount of particular SR proteins were detected. SRp75 is abundant in this preparation of SR proteins from thymus and other tissues, which suggests an apparent molecular mass change for the purified SRp70 protein that may reflect partial proteolysis or dephosphorylation of SRp75 during the purification. SR proteins appear to be most abundant in thymus extracts. Reflectance densitometry (*19*) indicates that the total signal due to mAb104 binding to thymus SR proteins is 6.5 times higher than that mea-

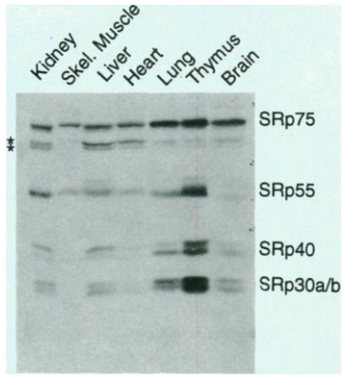

Fig. 4. SR proteins are differentially expressed in calf tissues. Immunoblot of proteins extracted from calf tissues with mAb104. Tissues were submerged in liquid N_2 in the slaughterhouse within 1 min after the animal was killed and subsequently powdered under liquid N_2 with a mortar and pestle. Frozen tissue powder was added to buffer containing 50 mM tris-Cl (pH 6.8), 20 mM EDTA, 5% SDS, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 5 mM β-glycerophosphate. The suspensions were sonicated three times for 30 s and then centrifuged at 20°C for 20 min at 13,000*g*. Glycerol and β-mercaptoethanol were added (to 10% and 2% final concentrations, respectively) to 200 μg of each extract per lane, and the samples were subjected to SDS-PAGE on a 10% acrylamide gel. After transfer to nitrocellulose, blots were incubated with either mAb104 or no primary antibody (as a control) (*3*). The appropriate positions for each SR protein are indicated. Note that SRp30a/b and SRp40 migrate as doublets; the nature of this heterogeneity is unknown. Two bands of 70-kD size were present in control immunoblots and therefore are marked with asterisks to indicate that they are nonspecific. Skel. Muscle, skeletal muscle.

sured for skeletal muscle, which gave the lowest signal. In addition, differences in the relative amounts of individual SR proteins correlated with tissue type. For example, SRp30a/b was most abundant in thymus extracts, whereas this band was nearly undetectable in extracts of either skeletal or cardiac muscle. In brain as well as in both muscle types, SRp75 accounted for about 50% of the total mAb104 signal per lane, as compared with 20% of the total in the thymus. SRp55 is only faintly detectable in brain extract in which SRp40 and SRp30a/b are also only minor components. In view of the heterogeneity of cell types in each tissue, it is possible that variations in the relative abundance of distinct SR proteins may be much greater in single cells. These results show that the differentiated state of cells is associated with the expression of a particular set of SR proteins.

Other results have shown that many different SR proteins isolated from calf thymus, HeLa cells, and cultured *Drosophila* cells have the same effect on the splicing of introns from pre-mRNAs in vitro (*1, 4, 5, 8*). The high degree of size and sequence conservation of the different SR proteins among species, however, suggests that individual SR proteins have distinct and essential functions in vivo. The findings described here show that particular members of the SR family have distinct functions in vitro. The observation that particular SR proteins are associated with the preferential use of different 5′ splice sites suggests that SR proteins may function in alternative splicing in vivo. The absolute and relative amounts of SR protein family members expressed by a given cell may determine the preferred splice site usage and therefore contribute to the regulation of gene expression.

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23. We thank J. Manley for providing splicing construct pSVi66; J. Wallace for sequence analysis; R. Robinson for protein digestion and separation; M. Gordy for peptide sequencing; L. Bohor-Roth, J. Priess, M. Groudine, and S. Hahn for careful reading of the manuscript; and R. Tuma for comments. Supported by grant 42786-02 (M.B.R.) from the National Institute for General Medical Sciences. A.M.Z. is a Burroughs-Wellcome Fund Fellow of the Life Sciences Research Foundation. K.M.N. is a Postdoctoral Fellow of the American Cancer Society.

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Spatially Resolved Dynamics of cAMP and Protein Kinase A Subunits in *Aplysia* Sensory Neurons

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Cyclic adenosine monophosphate (cAMP)–dependent protein kinase, labeled with fluorescein and rhodamine on the catalytic and regulatory subunits, respectively, was injected into *Aplysia* sensory neurons either in culture or in intact cell clusters. Energy transfer between the subunits, a measure of cytosolic cAMP concentration ([cAMP]), and compartmentation of the dissociated subunits were monitored by confocal fluorescence microscopy. Bath application of serotonin produced a much greater elevation of [cAMP] in the processes than in the central bodies of the neurons. The resulting gradients must drive a sizable centripetal flux of cAMP because direct microinjection of cAMP showed that it diffused readily. Perinuclear increases in [cAMP] slowly caused the translocation of the freed catalytic subunit into the nucleus to an extent proportional to the percentage of its dissociation from the regulatory subunit.

Serotonin leads to an increase in [cAMP] and can elicit both short- and long-term synaptic changes in the monosynaptic connection between sensory and motor neurons of the gill-withdrawal reflex in the marine snail *Aplysia* (*1, 2*). The distinction between short- and long-term processes in sensory neurons seems to derive in part from the ability of the modulatory transmitter to select between a program involving only a cytoplasmic mechanism of regulation by cAMP or the induction of additional mechanisms that alter cell state through the activation of transcriptional regulatory proteins in the nucleus, such as CREB (*3*). To determine where transmitter generates cAMP, what concentrations are attained, and how the cAMP signal reaches the nucleus, we injected and imaged cAMP-dependent protein kinase labeled with both fluorescein and rhodamine (FlCRhR) (*4*). This labeled enzyme allows both the dynamic measurement of free cAMP and the tracking of the regulatory (R) and the catalytic (C) subunits in single cells.

The FlCRhR was introduced by pressure microinjection into the cell bodies of sensory neurons that were either isolated in culture (*5*) or in excised pleural ganglia. Fluorescence imaging was performed with a laser-scanning confocal microscope specifically designed for high-speed emission ra-

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