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Nature of bacterial colonization influences transcription of mucin genes in mice during the first week of life

Anders Bergström[1*], Matilde B Kristensen[1], Martin I Bahl[1], Stine B Metzdorff[2], Lisbeth N Fink[3], Hanne Frøkiær[2] and Tine R Licht[1]

Abstract

Background: Postnatal regulation of the small intestinal mucus layer is potentially important in the development of adult gut functionality. We hypothesized that the nature of bacterial colonization affects mucus gene regulation in early life.

We thus analyzed the influence of the presence of a conventional microbiota as well as two selected monocolonizing bacterial strains on the transcription of murine genes involved in mucus layer development during the first week of life.

Mouse pups (N = 8/group) from differently colonized dams: Germ-free (GF), conventional specific pathogen free (SPF), monocolonized with either *Lactobacillus acidophilus* NCFM (*Lb*) or *Escherichia coli* Nissle (*Ec*) were analyzed by qPCR on isolated ileal tissue sections from postnatal days 1 and 6 (PND1, PND6) after birth with respect to: (i) transcription of specific genes involved in mucus production (*Muc1-4*, *Tff3*) and (ii) amounts of 16S rRNA of *Lactobacillus* and *E. coli*. Quantification of 16S rRNA genes was performed to obtain a measure for amounts of colonized bacteria.

Results: We found a microbiota-independent transcriptional increase of all five mucus genes from PND1 to PND6. Furthermore, the relative level of transcription of certain mucus genes on PND1 was increased by the presence of bacteria. This was observed for *Tff3* in the SPF, *Ec,* and *Lb* groups; for *Muc2* in SPF; and for *Muc3* and *Muc4* in *Ec* and *Lb*, respectively.

Detection of bacterial 16S rRNA genes levels above the qPCR detection level occurred only on PND6 and only for some of the colonized animals. On PND6, we found significantly lower levels of *Muc1*, *Muc2* and *Muc4* gene transcription for *Lb* animals with detectable *Lactobacillus* levels as compared to animals with *Lactobacillus* levels below the detection limit.

Conclusions: In summary, our data show that development of the expression of genes encoding secreted (*Muc2/Tff3*) and membrane-bound (*Muc1/Muc3/Muc4*) mucus regulatory proteins, respectively, is distinct and that the onset of this development may be accelerated by specific groups of bacteria present or absent at the mucosal site.

Keywords: Germ free mice, Monocolonized, qPCR, LinRegPCR, Postnatal transcription onset, Probiotics, *Lactobacillus acidophilus* NCFM, *Escherichia coli* Nissle, 16S rRNA

* Correspondence: adbe@food.dtu.dk
[1]Gut Ecology Group, Department of Food Microbiology, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, Søborg 2860, Denmark
Full list of author information is available at the end of the article

Background

The interplay between the microbiota of the gut and the intestinal mucus layer in early life is important in the development of the epithelial barrier as part of the innate immune defense [1]. The first weeks and months after birth are believed to be crucial for establishment of the gut microbiota and consequently for the health and integrity of the epithelium throughout life [2,3]. In this period, a development regulated by endogenous factors such as hormones, in parallel with gene regulation caused by the microorganisms present in the gut, takes place [4,5]. The presence and composition of the microbiota has been shown to be directly involved in the regulation of gene transcription in the intestinal epithelium, including the mucin genes, *Muc1-4* and the trefoil factor *Tff3* [4,6].

In the human intestines, MUC1-4 are the most prevalent [6] of the different mucin gene transcripts described to date [1,7,8]. In the gastrointestinal tract, specific mucins show coordinated expression and localization with the viscosity regulating trefoil factors (TFF's), in particular TFF3 [1]. Epithelial linings contain both membrane-bound (MUC1, MUC3, MUC4) and secreted gel-forming mucins (MUC2) expressing highly specific oligosaccharide side chains, which are important in relation to filtering the entry of various moieties e.g. bacteria and food to the underlying tissue. The membrane-bound mucins act as cell-surface receptors and sensors, mediating signals to trigger cell proliferation, apoptosis, differentiation and specific secretions, when relevant [1]. The four human mucin genes (*MUC1-4*) all share a fairly high degree of sequence, distribution and functional homology to the mouse mucin genes *Muc1-4* [9-12].

As facultative anaerobes, lactobacilli and *E. coli* strains have been recognized as successful early life colonizers of the sterile gastro-intestinal tract [13,14]. Strains of *Lactobacillus acidophilus* are known to stimulate transcription of mucin genes *in vitro* [15,16]. Moreover administrations of probiotic lactobacilli and bifidobacteria have been shown to increase ileal gene and protein levels of *Muc3* in adult rats [17] and cell cultures [16], respectively. Certain *E. coli* strains have been associated with increased production of *MUC2, MUC3* and *MUC4* in human ileal cells [18].

In order to elucidate the role of microbial colonization in the postnatal regulation of *Muc1-4* and *Tff3*, we investigated the expression of these genes in mouse ileal segments isolated at the first day after birth (PND1) and six days after birth (PND6), respectively, from specific pathogen free, conventionally bred mice (SPF), mice monocolonized with either *Lactobacillus acidophilus* NCFM (*Lb*) [19] or *E. coli* Nissle (*Ec*) [20,21], and from germ free (GF) mice [15,22]. Specifically, samples were collected and analyzed at PND1 and PND6 to examine the immediate postnatal effects, which are relevant for immune system priming [22,23]. Quantification of bacterial 16S rRNA gene levels was performed to obtain a measure of bacterial colonization levels in the different animal groups on PND1 and PND6.
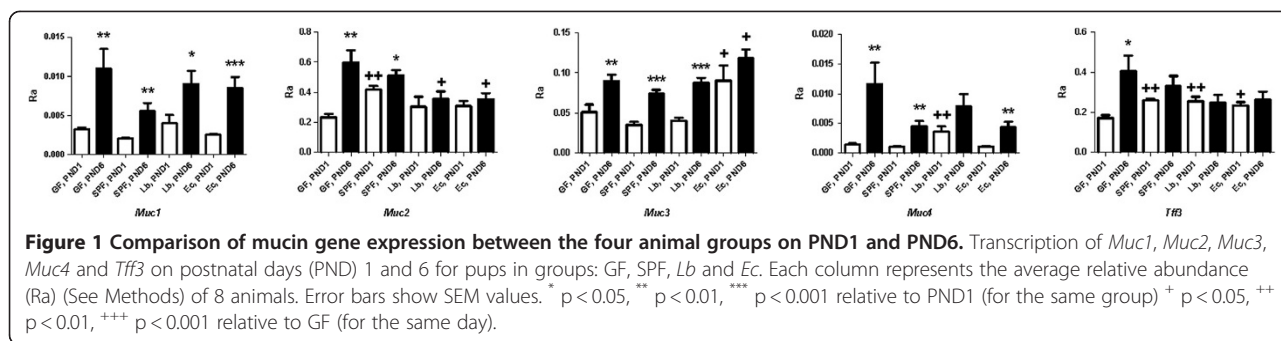
Results and discussion

qPCR

We introduced several new primers in this study, all scoring successfully on our validation criteria. LinRegPCR [24,25] was utilized for qPCR analysis, as it enables individual PCR efficiencies to be calculated. The standard curve assumption, that in all samples the PCR efficiency/amplicon, based on one "representative" DNA sample is constant, is replaced by an assumption-free method based on linear regression in the exponential phase of the fluorescence of the actual individual samples analyzed [24]. Further, by including in the subsequent calculation of average efficiency/amplicon, only successful samples within 5 % of the mean efficiency/amplicon, contributions from diverging samples to the final results are excluded.

We tested the choice of reference gene, but interestingly found no significant difference in the results between beta-actin [26,27], neuroplastin (Genevestigator recommendation) nor the geometrical mean of them both.

Effect of time and bacterial colonization on regulation of Muc1-4 and Tff3 transcription

In GF mice, *Muc1-4* and *Tff3*, all showed statistically significant increases in transcription from PND1 to PND6, indicating that this event occurs during the first postnatal week independently of the presence of microbes (Figure 1). For certain mucin genes, presence of bacteria in the colonized animals correlated with an increased relative abundance of transcripts on PND1 compared to transcription levels of the same genes in GF mice. This was particularly evident for the genes *Muc2* and *Tff3*. Increased transcription on PND1 of *Tff3* was observed in conventional pups (SPF) as well as in pups of dams' monocolonized with either *Lb* or *Ec*, while for *Muc2*, this was observed only in presence of a full microbiota (SPF). For *Muc3* in *Ec* and *Muc4* in *Lb*, a higher level of transcription was observed on PND1 than in GF pups, indicating that *E. coli* and *Lactobacillus* may specifically stimulate transcription of these genes immediately after birth (Figure 1).

The higher level of *Muc2* and *Tff3* transcriptions at PND1, both encoding secreted proteins with goblet cell origin [28], indicates that the presence of bacteria affects gene transcription onset in these exocytotic cells. While both gene products play protective roles during gut inflammatory conditions, at sites of epithelial damage [18,29-34] and during postnatal development [35,36],

Figure 1 Comparison of mucin gene expression between the four animal groups on PND1 and PND6. Transcription of *Muc1*, *Muc2*, *Muc3*, *Muc4* and *Tff3* on postnatal days (PND) 1 and 6 for pups in groups: GF, SPF, *Lb* and *Ec*. Each column represents the average relative abundance (Ra) (See Methods) of 8 animals. Error bars show SEM values. * p < 0.05, ** p < 0.01, *** p < 0.001 relative to PND1 (for the same group) + p < 0.05, ++ p < 0.01, +++ p < 0.001 relative to GF (for the same day).

Muc2, unlike Tff3, polymerizes into a protective gel-like structure [1]. Based on the obtained results, it is however not possible to determine whether there is a connection between this difference in functionality and the corresponding gene regulation.

Previously, we demonstrated how microbiota affects ileal gene expression of a number of immune related genes (specific cytokines and chemokines) during the first week after birth [23]. As seen for *Muc2* in the present study, and also for a number of Toll-like receptor signaling pathway related genes such as *Tlr2/4*, *Irak1* and the chemokine *Cxcl2*, encoding MIP-2, the presence of a full microbiota was required to influence gene expression on PND1, which was only to a limited degree affected by monocolonization with either *Lactobacilli* or *E. coli* [23].

Increased transcription of *Muc3* and *Muc4* on PND1 was observed in *Ec* and *Lb* pups, respectively, but not in SPF (Figure 1). Although specific probiotic bacteria, including *Lactobacillus acidophilus* NCFM [15], *Lactobacillus rhamnosus* [17], *Bifidobacterium bifidum* [17], *Lactobacillus plantarum* [16,17] as well as two atypical, enteropathogenic *E.coli* strains [18], have previously been shown to stimulate mucin gene expression, this study is to our knowledge the first to address such effects at a very early stage of life. *Muc1* transcription levels were in this study apparently not affected by the presence of bacteria.

Bacterial 16S rRNA abundance on PND1 and PND6

None of the PND1 samples contained *Lactobacillus* or *E. coli* in amounts above the qPCR detection limit (DL), in any of the four animal groups (Table 1). This was expected, since only partial bacterial colonization is achieved so short after birth. On PND6, 5/8 pups in both the *Lb* and SPF groups, respectively, were positively above the *Lactobacillus* 16S rRNA DL, while 8/8 and 0/8 in the *Ec* and SPF groups, respectively, were colonized above the *E.coli* DL. These observations corresponded to differences in N_0 values (See Methods) of >300-fold for *Lactobacillus* and >160-fold for *E.coli*. This shows that

bacterial levels in the ileal sections increased between PND1 and PND6 after birth, although the employed procedure did not allow detection of bacterial 16S rRNA in all pups. Culture-based techniques have shown that the gut mucosal surfaces in newborn mice follow a rather conserved colonization pattern [37]. In particular, lactobacilli colonize within the first 1–2 days after birth, whereas coliforms are normally not quantifiable in the mucosal layers until approximately 9 days after birth [14]. These results are thus consistent with findings in the SPF group in this study. It is however important to note, that the current analysis was performed on whole intestinal sections, including both luminal contents and mucosal surfaces, whereas the other studies referred to were based on analysis of mucosal surfaces only.

There was a significantly lower level of transcripts (p < 0.05) of *Muc1*, *Muc2* and *Muc4* in the pups with detectable amounts of lactobacilli on PND6 in the *Lb* group than in pups with colonization below the detection limit (Figure 2). In other words, colonization with relatively high levels of *Lactobacilli* in the pups had a negative effect on mucin gene transcription on PND6. For *Muc2*, pups colonized with *Lactobacillus* below the detection limit in the *Lb* group were indeed comparable to GF pups.

It has been established by others that degradation of mucin in adult rats [38] as well as gene expression of *Muc1-4* and *Tff3* in six week old mice [6], is different

Table 1 16S rRNA measured presence vs. absence of all 4 animal groups on each of days PND1 and PND6

	PND1		PND6	
	Lactobacillus 16S	*E. coli* 16S	*Lactobacillus* 16S	*E. coli* 16S
GF	0/8	0/8	0/8	0/8
SPF	0/8	0/8	5/8	0/8
Ec	0/8	0/8	0/8	8/8
Lb	0/8	0/8	5/8	0/8

The fraction denotes number of samples significantly above detection limit (DL) of the total number (N = 8 in each group).
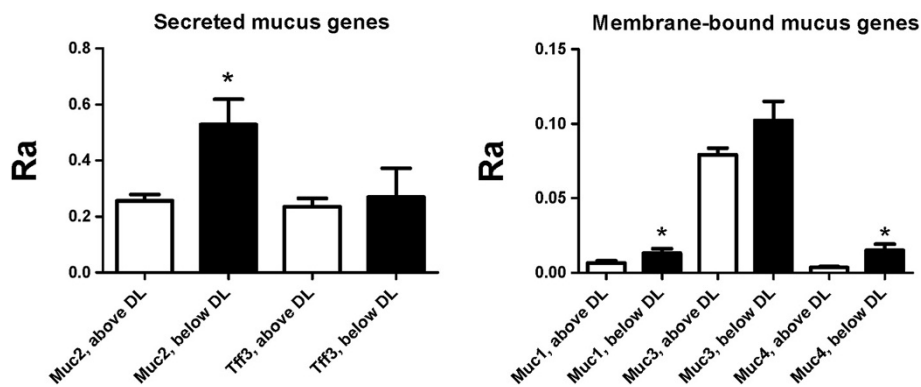
Figure 2 Difference in mucin gene expression between pups with detectable and undetectable *Lactobacillus* 16S in *Lb*. Comparison of *Muc1*, *Muc2*, *Muc3*, *Muc4* and *Tff3* transcription on PND6 between pups in the *Lb* group, where *Lactobacillus* 16S could be detected and could not be detected, respectively. Each column represents the average relative abundance (Ra) of 5/8 (above DL) or 3/8 (below DL) animals. Error bars show SEM values. DL = Detection Limit. * p < 0.05, denotes significant difference between detectably colonized and not colonized.

when comparing GF and conventional animals. Clearly, gene regulation induced by the colonizing microbiota is a complex and continuous process occurring throughout the first weeks of life, and as a more stable and adult-like microbiota is probably not achieved until the end of weaning process at approximately 21 days after birth [39], the expression of the mucus regulating genes may change not only in newborn animals, but also later in life in response to periodic changes in the microbiota

Conclusions

In this manuscript, we show distinct differences between the expression patterns of secreted (*Muc2*/*Tff3*) and membrane-bound (*Muc1*/*Muc3*/*Muc4*) mucus-regulatory genes in the very first days after birth. Presence of a full microbiota (SPF) increased the relative level of transcription of *Muc2* and *Tff3*, which implies the two corresponding secreted gene products, Muc2 and Tff3, to play protective roles in the postnatal intestinal layer development. The immediate activation of *Muc2*/*Tff3* transcription may provide a coating of the new born ileal epithelial layer, allowing only passage of certain substances or organisms.

Methods

Animal experiments

GF Swiss Webster mice and SPF mice, containing conventional microbiota, were purchased from Taconic (Lille Skensved, Denmark) and kept in GF isolators or under specific pathogen-free conditions, respectively [22]. Fecal samples from GF mice, taken at sampling i.e. once a week, were cultivated on non-selective LB medium and under aerobic and anaerobic conditions to confirm sterility of isolators. For breeding, pairs of female mice were housed with one male until plugs were observed. Mono-colonization of pregnant mice with *Ec* and *Lb* was

performed 7 days after mating by applying 5×10^{8} CFU ml^{-1} in 0.5 ml PBS suspension orally using a pipette and 0.5 ml to the abdominal skin. *Lb* was grown anaerobically in de Man, Rogosa, and Sharpe broth (MRS, Merck, Darmstadt, Germany) and *Ec* aerobically in Luria-Bertani broth (LB, Merck) overnight at 37°C. The cultures were harvested, washed twice in sterile phosphate-buffered saline (PBS) (Lonza, Basel, Switzerland), re-suspended in 1/50 of the original culture volume and frozen at –80 °C until use. Prior to administering bacteria to the mice, *Ec* suspensions were diluted tenfold in PBS immediately to obtain 5×10^{8} CFU ml^{-1}. *Lb* suspensions were not diluted. Four litters spontaneously delivered from 4 different mothers in each group; SPF, GF, *Lb* and *Ec*, were used for the experiment. At post-natal days 1 and 6, the pups were put down and the distal ileum (segment from cecum and 3 cm up) was removed from the small intestine of two pups per litter and frozen in RNAlater (Qiagen, Hilden, Germany). No separation of mucosal from luminal content was performed.

Ethics

The mouse experiment was performed under a license to Department of Microbiology, National Food Institute, from the Danish Council for Animal Experimentation.

RNA isolation

Tissues were removed from RNAlater and homogenized by a rotor strator in RLT buffer (Qiagen). RNA from tissue homogenate was extracted using RNeasy Mini Kit from Qiagen following the supplier's protocol.

Primer design and validation

A list of all primers used in this study is presented in Table 2. All primers found in references were initially checked with the Net Primer Software (http://www.

Table 2 Primers used for qPCR

Primer name	Fwd (5´-3´)	Rev (5´-3´)	Amplicon size	Reference
Muc1	TCGTCTATTTCCTTGCCCTG	ATTACCTGCCGAAACCTCCT	185	This study
Muc2	CCCAGAAGGGACTGTGTATG	TTGTGTTCGCTCTTGGTCAG	276	Modified from [44]
Muc3	TGGTCAACTGCGAGAATGGA	TACGCTCTCCACCAGTTCCT	98	Modified from [6]
Muc4	GTCTCCCATCACGGTTCAGT	TGTCATTCCACACTCCCAGA	280	This study
Tff3	CTCTGTCACATCGGAGCAGTGT	TGAAGCACCAGGGCACATT	77	[45]
Neuroplastin	CGCTGCTCAGAACGAACCAAGAA	CTTACGGGTGGCAGTGAGTT	160	Modified from [46]
Beta-actin	GTCCACCTTCCAGCAGATGT	GAAAGGGTGTAAAACGCAGC	117	This study
Lactobacillus 16S rRNA	AGCAGTAGGGAATCTTCCA[a]	CACCGCTACACATGGAG[b]	341	[a] [47][b] [48]
E. coli 16S rRNA	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA	96	[49]

premierbiosoft.com/netprimer/index.html). Primers not scoring a rating of at least 90 % were not accepted and new primers were then designed with NCBI's primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the quality was again verified until satisfaction with the Net Primer Software. All newly designed primers were designed to span exon junctions to avoid amplification of genomic DNA. The specificity of all primers was evaluated *in silico* using nucleotide BLAST, (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative PCR (qPCR)

Isolated ileal RNA was reverse transcribed into cDNA using SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen, Denmark. After verifying the quality of the cDNA by spectroscopy ($A_{260}/A_{280} = 1.8 \pm 10$ %) measured on a NanoDrop ND-1000 Spectrophotometer (Saveen Werner, Limhamn, Sweden), it was used as template in quantitative real-time PCR using the ABI prism 7900HT from Applied Biosystems. All cDNA concentrations were within the range of 90-100 ng μl^{-1}. The amplification reactions were carried out in a total volume of 20 µl containing 10 µl master mix (2x PerfeCTa™ SYBR® Green Super-Mix, ROX from Quanta Biosciences ™), 0.4 µl of each primer (10 µM), 2 µl template cDNA, and 7.2 µl nuclease-free water (Qiagen GmbH, Germany) purified for PCR. The amplification program consisted of one cycle of 50 °C for 2 min; one cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and finally one cycle of dissociation curve analysis for assessing the amplification products (95 °C for 15 s, 60 °C for 20s and increasing ramp rate by 2 % until 95 °C for 15 s). These conditions were selected based on preliminary qPCR experiments on target DNA with similar concentrations (100 ng μl^{-1}). Samples of all amplification products were further subjected to gel electrophoresis in 2 % agarose, followed by ethidium bromide staining in order to verify amplicon sizes.

qPCR setup

Three separate qPCR experiments on ileal cDNA were performed; 1) and 2) were separate replications of relative quantifications on mucus gene transcription (Muc1-4 and Tff3) with selected reference genes (see next paragraph) and 3) on presence or absence of specific bacterial 16S rRNA analysis (Lactobacillus, E.coli).

qPCR data analysis

All qPCR analysis was performed with the freely available LinRegPCR tool developed by Ruijter et al. [24,25]. The raw fluorescence data were exported from the ABI prism 7900HT SDS-software, and the LinRegPCR program was used to estimate baselines and individual PCR efficiencies in order to calculate output as target starting concentration, expressed in arbitrary fluorescence units N_0, for each PCR sample by the formula $N_0 = \text{threshold} / (\text{Eff}_{mean}^{Ct})$, where Eff_{mean} denotes the optimal PCR mean efficiency/amplicon, threshold the optimal "cutoff" in the exponential region and C_t is the number, where each PCR sample exceeds this threshold. Samples with no amplification, baseline error, too much noise or without plateau were automatically excluded by the LinRegPCR software. Subsequently, for each amplicon the average of all remaining, successful samples within 5 % of the mean value of all successful samples/amplicon were used in the calculation of Eff_{mean} for each amplicon. All N_0-values were calculated as means of double qPCR determinations.

For relative quantification of mucus gene transcripts, two different eukaryotic reference genes were used namely beta-actin [40] and neuroplastin, the latter suggested by the Genevestigator software (https://www.genevestigator.com) [41,42] based on microarray data on similar organism (*M. musculus*) and tissue (ileum). We used the geometric mean of the two reference genes as previously suggested [43]. Normalization to relevant reference gene expression was then calculated according to the formula:

$Ra = \text{Ratio} = N_0^{\text{Sample}} / N_0^{\text{Reference}}$ and averaged over the two qPCR experiments.

Unspecific amplification of 16S rRNA bacterial genes from GF mice was used to specify detection limits for specific amplifications (*Lactobacillus*, *E .coli*). Cutoffs for presence of either bacterium were defined by at least 5 C_t-values difference from the GF samples. No normalization to reference genes and thus relative quantification was used for the 16S analysis, since the purpose was only to determine presence vs. absence of detectable bacteria.

Statistics

All statistics was performed with GraphPad Prism 5. One-way ANOVA followed by Dunnett's *post hoc* test with GF as control group and Student's *t*-test was used to compare mucus gene expression between the four animal groups and development from PND1 to PND6, respectively. P-values lower than p = 0.05 were considered statistically significant. Welch's correction for unequal variances was applied, when necessary.

Authors' contributions
AB performed the qPCR experiments, including cDNA syntheses, data interpretation and statistical analysis, and wrote the manuscript. MBK and SBM performed the animal experiments, including isolation of ileal tissue and RNA purification. TRL, HF and LNF conceived of the study setup and participated in its design and coordination. TRL, MBK, HF and MIB contributed to data analysis and interpretation as well as preparation of the manuscript. All authors read and approved the final manuscript.

Author details
[1]Gut Ecology Group, Department of Food Microbiology, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, Søborg 2860, Denmark. [2]Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark. [3]Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark.

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