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Transferrin Receptor Gene Expression during Rat Liver Regeneration

EVIDENCE FOR POST-TRANSCRIPTIONAL REGULATION BY IRON REGULATORY FACTOR$_B$, A SECOND IRON-RESPONSIVE ELEMENT-BINDING PROTEIN*

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Transferrin receptor (TfR) expression is regulated by iron at the level of mRNA stability through a factor (IRF/IRE-BP) which binds to specific iron-responsive elements (IRE). On the other hand, growth-dependent regulation of TfR expression is generally believed to be transcriptionally controlled. We analyzed the molecular mechanisms that control TfR gene expression at the onset of cell proliferation *in vivo* during liver regeneration after partial hepatectomy. The amount of TfR mRNA increased considerably after partial hepatectomy while run-on assays did not show significant changes in TfR gene transcription. RNA band-shift assays documented a significant activation of IRF/IRE-BP specific for the faster migrating IRE-protein complex (IRF$_B$). These changes occurred in the absence of modifications of total liver iron concentration but together with a significant decrease of ferritin content. Moreover, when extreme variations of liver iron content were achieved by either chronic iron overload or severe iron deficiency, liver regeneration was unable to influence IRE-binding activity. We conclude that IRF/IRE-BP-mediated post-transcriptional control can fully account for TfR mRNA induction during liver cell proliferation *in vivo*. IRF/IRE-BP activation in the absence of changes in total tissue iron content might depend either on a drop of iron levels into the regulatory pool or on a relatively iron-independent mechanism specific for the faster migrating complex.

The expression of transferrin receptor (TfR),[1] which mediates iron uptake in most eukaryotic cells (1), is determined not only by cellular iron requirements but also by cell growth and differentiation, being generally tightly associated with cell proliferation (2–5). Changes in TfR gene expression in response to variations in intracellular iron levels are post-transcriptionally regulated through the interaction of an iron-dependent cytosolic protein (IRF/IRE-BP) with specific iron-responsive elements (IRE) in the 3′-untranslated region of TfR mRNA (6–9). In rodents, two IRE-protein complexes can form (10, 11), and a second IRE-binding protein, called IRF$_B$, has been recently characterized (12, 13). IRE sequences are also present in the mRNA of ferritin H and L subunits (14) and of erythroid aminolevulinate synthase (15, 16). Thus, proteins involved in intracellular iron metabolism are coordinately controlled by the action of a single iron-sensitive effector. In the case of TfR, changes in the affinity of IRF/IRE-BP for IRE rapidly alter the stability of TfR mRNA (17, 18). On the contrary, changes in TfR expression induced by alterations in the growth rate of cultured cells are believed to be transcriptionally regulated (3, 19–22).

To study the regulation of TfR gene expression during cell proliferation *in vivo* we used regenerating liver as a model system. In fact, an increase in the TfR population has been reported to occur in regenerating liver (23–25). In this work we studied TfR gene expression during liver regeneration by measuring steady-state levels and transcription rates of TfR mRNA as well as IRF/IRE-BP activity. To understand the role of iron in the control of TfR expression during liver regeneration we also induced cell proliferation in the livers of iron-loaded and iron-deficient rats and compared the molecular mechanisms of TfR expression with both liver iron concentration and ferritin content.

MATERIALS AND METHODS

Animals—Male albino rats maintained in a temperature-controlled room with a 0700–1900-h photoperiod and fed a standard diet *ad libitum* were used throughout. Liver regeneration was induced by 70% partial hepatectomy as described in Higgins and Anderson (26) or by administration of 0.5 ml/100 g of body weight of CCl_4. Sham operation consisted in laparatomy and liver manipulation under ether anesthesia. Iron overload was achieved by feeding a standard diet supplemented with 2.5% (w/w) carbonyl iron (GAF Corp., New York) as described elsewhere (27). Iron deficiency was induced in rats by rearing nursing dams and their offspring on an iron-free diet and by repeated bleeding.

Determination of Liver Iron Content—Liver iron content was determined by atomic absorption spectroscopy as previously described (27).

RNA Isolation and Northern Blot Analysis—Aliquots of 40 μg of total liver RNA were subjected to Northern blot analysis as previously described (28). Probes were ^{32}P-labeled, gel-purified DNA fragments excised from the mouse TfR cDNA clone pTfR-2 (29), the mouse c-*myc* pc54 (30), the pHcGAP clone for GAPDH (31). For quantitative determinations, autoradiograms were scanned with densitometer and the values were calculated by normalizing to the signal of the GAPDH probe.

Nuclear Transcription Assays—Liver nuclei were prepared and incubated for *in vitro* transcription as previously reported (32). Equal amounts of trichloroacetic acid-precipitable radioactivity for the samples to be compared were hybridized to an excess of filter-bound DNA probes as described in Cairo and Lucchini (33). In addition to the probes used for Northern blot analysis, the cDNAs used were the rat albumin RSA 57 clone (34) and the rat ferritin L subunit pRLFL3 clone (35).

RNA-Protein Gel Retardation Assays—Cytoplasmic extracts were prepared in the buffer described by Leibold and Munro (10), and 1-μg protein samples, as determined by the Bio-Rad protein assay, were incubated with an excess of ^{32}P-labeled IRE-containing RNA transcribed *in vitro*. Binding, in the presence or absence of 2% 2-mercapto-

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[1] The abbreviations use are: TfR, transferrin receptor; IRF, iron-regulatory factor; IRE, iron-responsive element; BP, binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

ethanol, incubation with heparin, RNase T1 digestion, and electrophoresis of RNA-protein complexes were performed as described elsewhere (36). To quantify IRF/IRE-BP activity, regions from dried gels corresponding to the fast migrating RNA-protein complex were cut, and radioactivity was determined by liquid scintillation counting.

In Vitro RNA Transcription—The pSPT-fer plasmids containing the IRE of human ferritin H chain (11) and a plasmid containing the 5′ end of the rat ferritin L chain pRLFL3 clone (35), which lacks IRE sequences, were transcribed *in vitro* with T7 RNA polymerase. Radioactive transcripts were synthesized with 100 μCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham Corp.).

Determination of Liver Ferritin Content—An aliquot of the cytoplasmic extracts used for determination of IRF/IRE-BP activity was heated at 70 °C for 10 min, cooled on ice, and centrifuged for 15 min at 15,000 × *g* and 4 °C. The supernatant was collected, and equal amounts of proteins, as determined by the Bio-Rad protein assay, were loaded on 7.5% acrylamide nondenaturing gels. Ferritin content was evaluated by densitometry of silver-stained gels.

RESULTS

Liver Regeneration Enhances TfR mRNA Levels—Previous studies documented an increase in TfR number on the surface of hepatocytes during rat liver regeneration (23–25). To examine the molecular mechanisms of this activation, RNA from rat livers at various times after PH was hybridized with a TfR cDNA probe. TfR mRNA was expressed at low levels in control liver, increased progressively reaching a maximum at 16 h, and returned to the uninduced level at 24 h (Fig. 1). Densitometric quantification indicated a 10-fold increase at 16 h (Table I). As a control, the same blots were sequentially hybridized with a probe for c-*myc* and for GAPDH. As previously demonstrated (37), c-*myc* mRNA was strongly but transiently induced by PH, whereas the expression of the GAPDH gene, which is known to be equally expressed in resting and proliferating cells (38), remained constant in all the samples. Increased TfR gene expression was obtained also when liver regeneration was induced by administration of a toxic dose of CCl_4 (data not shown). In the livers of sham-operated rats TfR gene expression increased with a time course similar to that of liver regeneration, but the extent of increase was much lower (Table I).

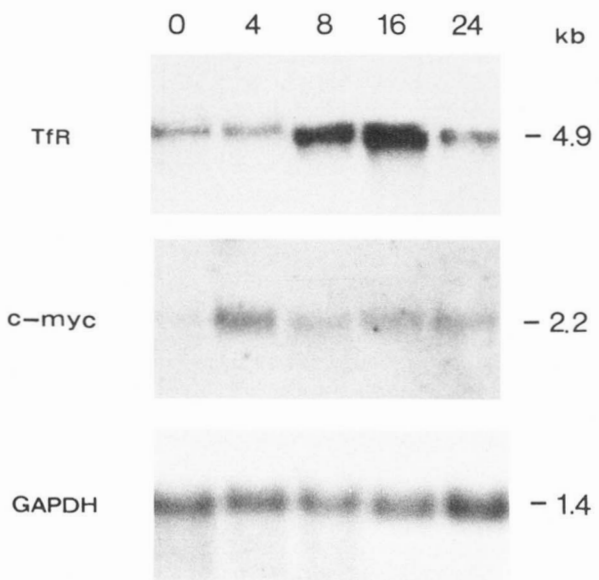

FIG. 1. **Northern blot analysis of TfR mRNA levels during liver regeneration.** Total RNA extracted from rat livers at different times after partial hepatectomy was hybridized to the indicated probes as described under "Materials and Methods." The autoradiogram shown is representative of three separate experiments.

TfR Gene Transcription Is Not Modified during Liver Regeneration—To investigate whether the activation of TfR mRNA after PH is transcriptionally regulated, nuclear run-on assays were performed. The transcription rate of TfR mRNA did not show significant variation (Fig. 2 and Table I), whereas that of other genes known to be transcriptionally regulated during liver regeneration (39, 40) was strongly affected. In fact, c-*myc* and albumin transcripts increased or decreased, respectively, at early times after PH and returned to normal levels later. Transcription of the control gene GAPDH remained constant in all of the samples.

Activation of IRF/IRE-BP during Liver Regeneration—The finding of strong differences in TfR mRNA steady-state levels in the absence of significant changes in TfR gene transcription prompted us to test the role of IRF/IRE-BP in the induction of TfR gene expression in regenerating livers. Since IRF/IRE-BP appears to be controlled at the post-translational level (6–9), the evaluation of its function is made by an activity assay (*i.e.* RNA band shift assay) rather than by quantitative assessment of protein levels. The RNA-gel-retardation assay illustrated in Fig. 3 shows that the IRE probe forms two distinct complexes with cytoplasmic extracts from rat liver. Competition studies showed that both bands are specific IRE-protein complexes since binding was prevented by competition with homologous unlabeled RNA, whereas an unrelated RNA lacking IRE was uneffective (Fig. 3, *Panel C*). However, only the fast migrating band was responsive to the various treatments, the upper band

TABLE I

TfR mRNA levels and transcription rate in relation to IRF/IRE-BP activity, hepatic iron, and ferritin content

The values of TfR mRNA steady-state levels were calculated as described under "Material and Methods" after normalization for the amount of GAPDH mRNA. TfR gene transcription was evaluated by densitometric scanning of autoradiograms. Spontaneous IRF/IRE-BP activity was determined by liquid scintillation counting of regions of the gel corresponding to the fast migrating protein/RNA complex. Ferritin content was evaluated by densitometric scanning of silver stained gels. Values for TfR mRNA, TfR transcription IRF/IRE-BP activity, and ferritin content represent mean ± S.D. of at least three separate experiments and are given as a percentage of the control.

Groups[a]	TfR mRNA	TfR transcription	IRF activity	Liver ferritin	Iron
	%	%	%	%	μg/g dry wt
Control	100	100	100	100	260 ± 68
PH 4	97 ± 8	106 ± 7	N.D.[b]	56 ± 3	241 ± 55
PH 8	245 ± 15	97 ± 10	180 ± 12	19 ± 6	253 ± 31
PH 16	970 ± 85	109 ± 18	350 ± 42	46 ± 4	285 ± 43
PH 24	180 ± 23	102 ± 14	97 ± 10	44 ± 5	214 ± 51
SH 4	105 ± 21	ND	ND	ND	180 ± 56
SH 8	130 ± 14	ND	ND	103 ± 7	197 ± 66
SH 16	290 ± 42	104 ± 13	145 ± 22	92 ± 6	160 ± 49
SH 24	120 ± 28	ND	ND	98 ± 4	232 ± 21
I.O	5 ± 3	95 ± 8	3 ± 2	350 ± 27	4610 ± 326
I.O. + PH 16	7 ± 4	101 ± 10	5 ± 3	ND	3215 ± 221
I.D.	2050 ± 154	114 ± 21	2650 ± 300	12 ± 3	60 ± 25

[a] PH: 4, 8, 16, 24 = 4, 8, 16, 24 h after partial hepatectomy; SH: 4, 8, 16, 24 = 4, 8, 16, 24 h after sham operation; I.O., iron overload; I.D., iron deficiency.

[b] ND, not determined.

being rather invariant. The RNA binding activity increased during liver regeneration with a peak at 16 h, well above the level of both control and sham-operated rats (Fig. 3, *Panel A*, and Table I). In agreement with previous reports (12, 13), preincubation of the extracts with 2-mercaptoethanol only partially increased the IRE binding activity (Fig. 3, *Panel B*), therefore only spontaneous activity was taken into account for quantitative evaluations (Table I). Enhanced IRF/IRE-BP activity was found also in CCl_4-treated rats (data not shown).

Effect of Alterations in Cellular Iron Content on the Regulation of TfR Gene Expression during Liver Regeneration—To understand whether the regulation of TfR induction in cell proliferation can be explained only by iron consumption or whether other mechanisms also play a role, we studied the effect of PH on TfR expression in the livers of iron-loaded and iron-deficient rats. In the livers of rats chronically fed an iron-enriched diet which presented a massive siderosis, the steady-state level of TfR mRNA was severely down-regulated (Table I). PH in iron-loaded rats was not able to induce TfR gene expression which, 16 h after the operation, remained below the level of control liver. On the contrary, a dramatic activation of TfR mRNA expression was found in rats with low intracellular iron levels (Table I). In these animals, PH did not show any additive stimulatory effect on the level of TfR mRNA (data not shown). With these experimental settings, we did not find significant changes in TfR gene transcription (Table I). Determination of IRF/IRE-BP activity by gel-shift assay (Table I) revealed that, in iron-overloaded animals, formation of the faster migrating complex was dramatically repressed and was not activated after PH. On the contrary, a very high IRF/IRE-BP activity was

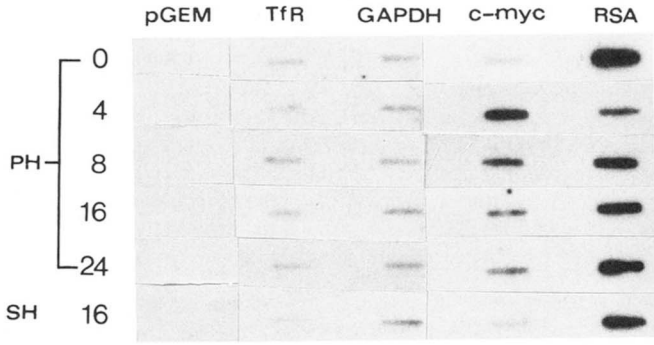

FIG. 2. **TfR gene transcription during liver regeneration.** Equal amounts of radioactive RNA transcribed *in vitro* from nuclei isolated from rat livers at different times after partial hepatectomy or at 16 hours after sham operation was hybridized to the indicated DNA probes immobilized on nitrocellulose filters. The autoradiogram shown is typical of three separate experiments.

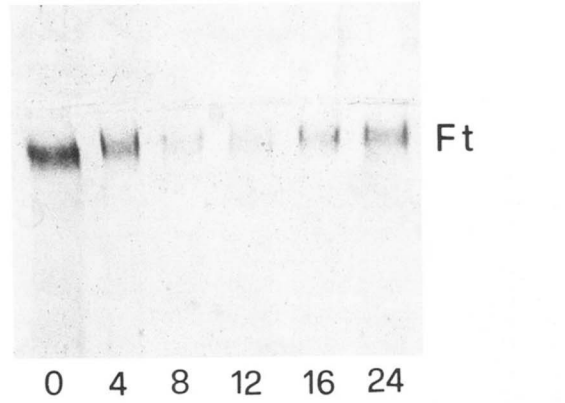

FIG. 4. **Ferritin content during liver regeneration.** Aliquots of cytoplasmic extracts used for band shift assay were enriched in ferritin by heating as described under "Materials and Methods." Equal amounts of proteins (5 μg) were loaded on nondenaturing 7.5% polyacrylamide gels. Ferritin was revealed by silver staining. The gel is representative of three separate experiments.

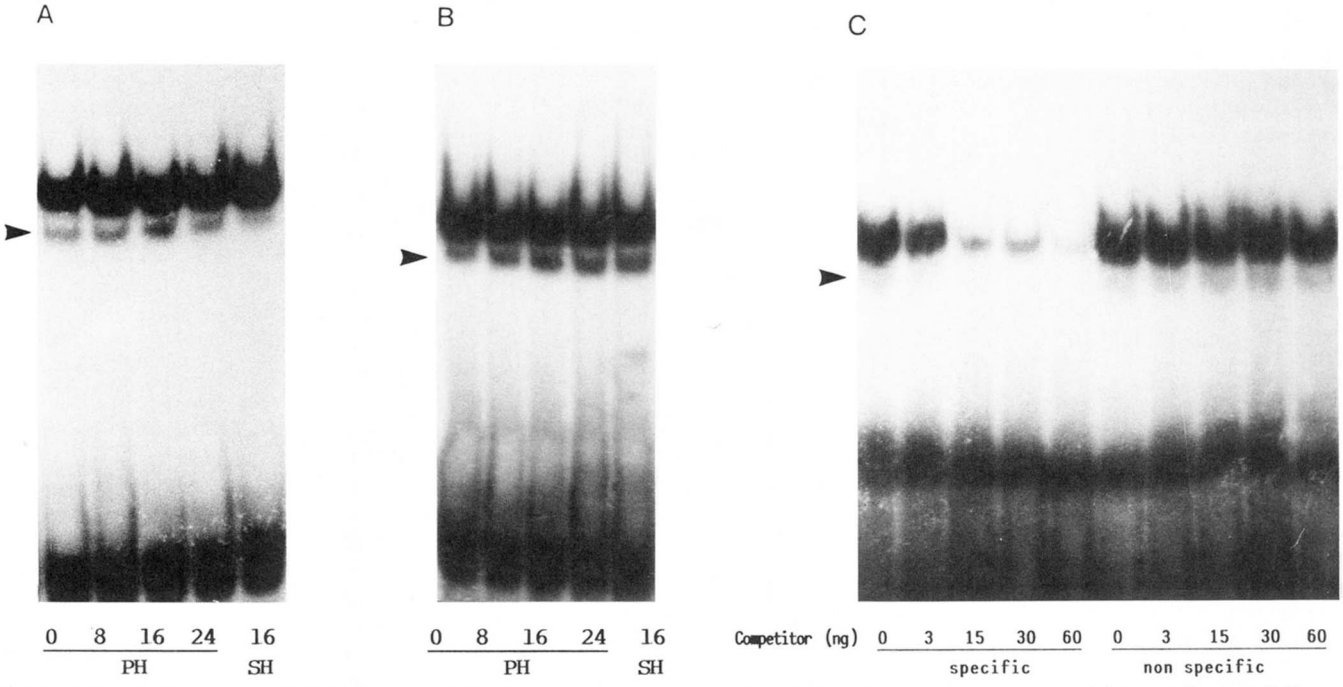

FIG. 3. **Band-shift assay of IRF/IRE-BP activity in rat liver.** *Panels A* and *B*, cytoplasmic extracts were prepared from rat livers at different times after PH or at 16 h after sham operation (*SH*). An excess of radioactive RNA transcribed from the pSPT-fer plasmid was added to an equal amount of proteins from each extract; after addition of RNase T1 and heparin, RNA-protein complexes were resolved on nondenaturing 6% polyacrylamide gel. The assay was performed in the absence (*Panel A*) or presence (*Panel B*) of 2-mercaptoethanol. The *arrowhead* indicates the faster moving complex which varies in the different samples. *Panel C*, cytoplasmic extracts from control rat liver were treated as described above but in the presence of increasing amounts of unlabeled RNA transcribed from the pSPT-fer (*specific*) or IRE-free ferritin (*nonspecific*) plasmids. The data shown are representative of three independent experiments.

found in liver extracts from iron-deficient rats.

Iron and Ferritin Content during Liver Regeneration—To assess the role of iron in the induction of TfR during liver cell growth, we measured the intracellular iron content by atomic absorption spectroscopy. Liver regeneration did not appreciably affect the hepatic level of the metal, whereas values considerably above or below normal levels were found in iron-loaded and iron-deficient animals, respectively (Table I). Taking advantage of the high thermal stability of ferritin, we also measured liver ferritin content by nondenaturing polyacrylamide gel electrophoresis and silver staining (Fig. 4). The assay was able to reveal the expected changes in liver ferritin content in iron-overloaded and iron-deficient rats (Table I). Liver ferritin content decreased progressively during liver regeneration to reach about 20% of the normal levels at 8–12 h. Later on, the amount of protein increased to some extent but remained below normal values (Table I). Similar variations in the amount of ferritin were observed in the livers of CCl_4-treated rats (data not shown), but not in sham-operated animals (Table I).

DISCUSSION

TfR gene expression can be regulated either post-translationally by redistribution of molecules between cytosol and the cell surface or at the level of mRNA accumulation (2). The present study shows that the amount of TfR mRNA increases during *in vivo* proliferation of hepatic cells. This suggests that the increased number of cell surface receptors previously reported (23, 24) is due not only to the redistribution of receptor molecules from internal pools (25), but also to increased synthesis of TfR mediated by higher levels of transcript.

The level of TfR mRNA has been shown to be modulated post-transcriptionally by iron levels and transcriptionally by variations in cell growth rate and/or differentiation (2, 3, 19, 41). The run-on transcription experiments reported in this work indicated that in rat liver the TfR gene is transcribed at the same rate under all experimental settings. Thus, it seems that, contrary to what has been described for TfR gene expression during growth activation *in vitro* (3, 20), transcriptional control is not involved in the activation of TfR during hepatocyte proliferation *in vivo*. Indeed, the finding that the activity of IRF/IRE-BP increases during liver regeneration in parallel with the rise of TfR mRNA suggests that the growth-dependent induction of TfR mRNA *in vivo* is post-transcriptionally regulated by this TfR mRNA-binding factor, as already reported during growth activation of spleen cells and T lymphocytes (42–44).

Activation of TfR at the onset of cell growth is considered to be due to increased iron consumption. However, the relation between cell growth and iron metabolism remains poorly defined. Present data show that during liver regeneration IRF/IRE-BP activation occurs without changes in total tissue iron concentration. Two alternative hypothesis can be put forward to interpret this apparent iron-independent activation of IRF/IRE-BP: it may either be caused by changes in intracellular distribution leading to reduced iron availability in the regulatory pool or by direct iron-independent regulation. As to the first hypothesis, the dramatically increased synthesis of iron-containing enzymes to sustain cell growth might subtract iron from the cytoplasmic regulatory pool and cause IRF/IRE-BP activation. In this context, the rapid decrease of liver ferritin content during liver regeneration could be part of a combined response of the hepatocyte that, needing iron for cell duplication, not only activates TfR gene expression to get iron from the circulation, but, as previously suggested (45), also rapidly degrades ferritin shells. However, the enhanced availability of free iron from this intracellular iron-rich source does not seem sufficient to compensate for the increased demand and to prevent IRF/IRE-BP activation.

On the other hand, the finding that, during liver regeneration, significant changes in IRF/IRE-BP activity only affect the faster migrating complex allows an alternative interpretation to be proposed. This complex (IRF_B), which is characteristic of rodents, represents the specific interaction between IRE and an iron-regulated protein structurally related to, but distinct from, IRF/IRE-BP (12, 13). In fact, it has tissue-specific abundance distribution (13), resistance to activation by reducing agents (12, 13) (Fig. 3) and lack of aconitase activity (12). In this work we show that only this IRE-binding protein is specifically activated by growth-related stimuli. Since activation during liver regeneration occurs in the absence of changes in total tissue iron content, one might suppose that IRF_B is preferentially activated by proliferative stimuli and is relatively iron-independent. However, since the formation of this complex is modulated also by iron levels (10–13), extreme conditions of cellular iron availability (*i.e.* iron overload or iron deficiency) probably exert a functional block that overcomes the variations induced by growth-related stimuli.

Present data showing specific activation of the faster migrating IRE-protein complex during *in vivo* cell proliferation provide the first evidence of a preferential modulation of the second IRE-binding protein and might be of particular interest as they further support the hypothesis that this factor might have an independent regulation and, along with TfR and ferritin regulation, might exert additional, still unrecognized, effects.

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