Translational Control of Collagen Prolyl 4-Hydroxylase-α(I)
Gene Expression under Hypoxia*

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Hypoxia is a pro-fibrotic stimulus, which is associated with enhanced collagen synthesis, as well as with augmented collagen prolyl 4-hydroxylase (C-P4H) activity. C-P4H activity is controlled mainly by regulated expression of the α C-P4H subunit. In this study we demonstrate that the increased synthesis of C-P4H-α(I) protein in human HT1080 fibroblasts under long term hypoxia (36 h, 1% oxygen) is controlled at the translational level. This is mediated by an interaction of RNA-binding protein nucleolin (~64 kDa form) at the 5′- and 3′-untranslated regions (UTR) of the mRNA. The 5′/3′-UTR-dependent mechanism elevates the C-P4H-α(I) expression rate 2.3-fold, and participates in a 5.3-fold increased protein level under long term hypoxia. The interaction of nucleolin at the 5′-UTR occurs directly and depends on the existence of an AU-rich element. Statistical evaluation of the ~64-kDa nucleolin/RNA interaction studies revealed a core binding sequence, corresponding to UAAUC or AAACU. At the 3′-UTR, nucleolin assembles indirectly via protein/protein interaction, with the help of another 3′-UTR-binding protein, presumably annexin A2. The increased protein level of the ~64-kDa nucleolin under hypoxia can be attributed to an autocatalytic cleavage of a high molecular weight nucleolin form, without alterations in nucleolin mRNA concentration. Thus, the alteration of translational efficiency by nucleolin, which occurs through a hypoxia inducible factor independent pathway, is an important step in C-P4H-α(I) regulation under hypoxia.

Collagen prolyl 4-hydroxylase (C-P4H),2 an α2/β2 tetramer, plays a central role in collagen synthesis. 4-Hydroxyproline residues are essential for the formation of triple-helical collagen molecules. The quantity and activity of C-P4H affects the composition of the extracellular matrix, because collagens constitute the major compound of extracellular matrix proteins. The collagen hydroxylation requires iron ions (Fe2+), 2-oxoglutarate, and oxygen (O2) (1). Ascorbate is essential to maintain the iron ions in their biologically active Fe2+ form. Three different α-subunit containing C-P4Hs are known, resulting in the formation of three isoenzymes called C-P4H (I), (II), and (III) (2). The β-subunit is identical to the enzyme and chaperone protein-disulfide isomerase (1, 2), and is required to keep the α-subunit in its soluble form (3). The α-subunits contain the catalytical domains of the tetramer and are limiting in the formation of active P4Hs. Thus, P4H activity appears to be mainly regulated by the amount of the α-subunit (4). The type (I) enzyme is the most abundant form of enzyme in most cells, except in chondrocytes and endothelial cells (5). However, enzymatic properties of types I–III isoenzymes are very similar (6, 7).

In addition to C-P4Hs, a second class of prolyl hydroxylases exist: the hypoxia inducible factor prolyl hydroxylases (HIF-PHs). They exclusively hydroxylate the transcription factor HIF (8). Both families of PHs depend on oxygen, which is of tangible importance in various physiological (e.g. altitude) and pathophysiological (e.g. ischemia) settings. The mechanisms by which hypoxia induces gene transcription is well established (9). Hypoxia reduces activity of HIF-PHs that hydroxylate specific proline residues in the oxygen-dependent degradation domain of the HIF-1α subunit. As a consequence, HIF-1α accumulates and promotes hypoxic tolerance by activating gene transcription (10). The C-P4H-α(I) gene (P4HA1) is one of the genes, which is transcriptionally activated via HIF (11), but additionally, as postulated (12) and as we investigated in detail in this work, it is also controlled posttranscriptionally. There seems to be a certain similarity to the collagens, which are also regulated at the posttranscriptional level (13). This coincidence may constitute a link between collagens and P4H as a basis of their co-regulation in collagen metabolism.

Long term or chronic hypoxia is a strong stimulus for collagen synthesis resulting in organ fibrosis, in particular in heart and liver (14, 15). The accumulation of collagens in the extracellular matrix following hypoxia is mediated by transforming growth factor-β (16–18), often associated with mRNA-specific posttranscriptional control (13). Posttranscriptional regulation, i.e. changes in mRNA stability or translational efficiency, is mainly attributed to the untranslated regions (UTRs) of mRNAs (19–21).
5′/3′-UTR Interaction of C-P4H-α(I) mRNA

The alteration of gene expression at the posttranscriptional level under stress conditions (22–24), in particular by hypoxia, has been demonstrated for several genes including collagens (13), vascular endothelial growth factor (22–24), erythropoietin (22–24), and tyrosine hydroxylase (25). Recently it was shown that in response to hypoxia, more genes are regulated at the level of translation than by changes in transcription rates (26). Recent studies revealed that C-P4H-α(I) mRNA may belong to a subclass of transcripts that are characterized by an increased translational efficiency under hypoxia (11, 12). The aim of this study was to analyze the molecular mechanism in the regulation of C-P4H-α(I) expression under hypoxia. In particular, we addressed the role of the 5′- and 3′-UTRs of C-P4H-α(I) mRNA in this process.

EXPERIMENTAL PROCEDURES

Cell Culture and RNA/Protein Isolation

Human fibrosarcoma HT1080 (ATCC, passages 16–21) cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose; PAA Laboratories GmbH), supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 15 mM Hepes, and 2 mmol/liter glutamine, at 37 °C, 5% CO2. Before use in experiments, cells were maintained in a medium containing 0.4% fetal calf serum for at least 24 h. Measurements started with the application of fresh medium containing 0.4% fetal calf serum. For hypoxic conditions the cells were incubated in a hypoxic chamber (JOUAN IG750). Oxygen content was reduced to 1% by gas exchange with 95% nitrogen, 5% CO2. Control cells were incubated under atmospheric oxygen conditions (21% O2, 5% CO2, 37 °C). To inhibit translation, cells were incubated in the presence of cycloheximide (20 μg/ml). For RNA and protein isolation, cells were washed with ice-cold phosphate-buffered saline. RNA was prepared using RNA-Bee (Biozol Diagnostica Vertrieb GmbH) according to the manufacturer’s protocol. Protein extracts (10,000 g supernatants, S10) were isolated using lysis buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 25% glycerol, 0.1% SDS, 0.5% Nonidet P-40, 200 μg/ml RNase Out (Invitrogen), 20 μg/ml cycloheximide, 1× Complete protease inhibitor mixture (Roche Diagnostics)). After a 10-min incubation on ice cells were centrifuged at 1,000 × g at 4 °C for 10 min. Supernatants were subjected to ultracentrifugation at 100,000 × g at 4 °C, for 2 h. Sediments represent a translationally active fraction. Supernatants represent a polysome-free fraction. After differential centrifugation, RNA was extracted from sediments and supernatants using RNA-Bee and analyzed by Northern blotting. For determination of protein localization, sediments were resolved in equal amounts of lysis buffer 2, and analyzed by Western blotting.

Western Blotting

Protein extracts (30 μg/sample) were separated by SDS-PAGE. After electrophoresis, proteins were transferred to Hybond™-P membranes (Amersham Biosciences) using a Bio-Rad Mini Trans-Blot transfer cell. The membranes were blocked for 1 h with 5% Blot-Quick Blocker (Chemicon). Following the blocking step, the membranes were incubated in 1% blocking solution containing a primary antibody (anti-P4H-α antibody, Acris Antibodies GmbH; anti-nucleolin antibody, Santa Cruz Biotechnology Inc.; anti-annexin A2 antibody, Acris Antibodies GmbH) at room temperature for 1.5 h or overnight at 4 °C. The membranes were washed three times with Tris-buffered saline with Tween 20 and incubated with a secondary antibody (anti-mouse, Promega) for 1 h. After additional washing steps bands were detected using the ChemiGlow™-West Detection Kit (Alpha Innotech Corporation). Membranes were stripped for 5 min at 4 °C, for 2 h. Sediments were subjected to differential centrifugation at 100,000 × g at 4 °C, for 2 h. Sediments represent a polysome-free fraction. After differential centrifugation, RNA was extracted from sediments and supernatants using RNA-Bee and analyzed by Northern blotting. For determination of protein localization, sediments were resolved in equal amounts of lysis buffer 2, and analyzed by Western blotting.

mRNA Quantification

RT-PCR—mRNA levels quantified by RT-PCR were normalized to relative β-actin levels. Primers were designed to bridge at least one intron. PCR conditions were used as follows: 3 min at 95 °C, cycles were 30 s at 95 °C; 30 s of annealing, 30 s at 72 °C, final elongation was for 2 min at 72 °C; 2.5 mM MgCl2. The primers were as follows: C-P4H-α(I), forward, 5′-CCACAGC-AGAGGAATTACAG, reverse, 5′-ACAAGCTGTTCAAACTTTTAGG; β-actin, forward, 5′-TGAAGTGCTAGTTGAGACGATC, reverse, 5′-CTAGATCCGTCTAGAAGGC; nucleolin, forward, 5′-AGACAGAGCTGATGAGAGG, reverse, 5′-TGTTGACTGGTAGGAGGT.

Northern Blotting—Isolated RNA was separated by electrophoresis on 1% agarose gels containing formaldehyde. The RNA was capillary transferred to positively charged nylon membranes (Roche Diagnostics), visualized after ethidium bromide staining to document the relative level of 18 S and 28 S rRNA, and hybridized to digoxigenin-labeled partial C-P4H-α(I) antisense transcripts (1,600 nt, representative for the coding region). The detection was performed using the digoxigenin RNA Labeling Kit (Roche Diagnostics) according to the manufacturer’s protocol. mRNA levels were normalized to 18 S/28 S rRNA.

Estimation of mRNA Stability

mRNA stability assays were performed as described in Ref. 27.

Differential Centrifugation

For investigation of mRNA and protein localization, cells were incubated in the presence of cycloheximide (20 μg/ml) for 10 min. Cells were washed in ice-cold phosphate-buffered saline supplemented with cycloheximide (20 μg/ml), harvested, and cell extracts were prepared using lysis buffer 2 (20 mM Tris, pH 7.5, 150 mM KCl, 25 mM MgCl2, 0.25% Nonidet P-40, 200 units/ml RNase OUT (Invitrogen), 20 μg/ml cycloheximide, 1× Complete protease inhibitor mixture (Roche Diagnostics)). After a 10-min incubation on ice cells were centrifuged at 1,000 × g at 4 °C for 10 min. Supernatants were subjected to ultracentrifugation at 100,000 × g at 4 °C, for 2 h. Sediments represent a translationally active fraction. Supernatants represent a polysome-free fraction. After differential centrifugation, RNA was extracted from sediments and supernatants using RNA-Bee and analyzed by Northern blotting. For determination of protein localization, sediments were resolved in equal amounts of lysis buffer 2, and analyzed by Western blotting.

Molecular Cloning and in Vitro Transcription

Partial C-P4H-α(I) sequences (GenBank™ gi:63252885), representing the C-P4H-α(I) 5′-UTR (133 nt) and 3′-UTR (999 nt) were amplified by PCR, cloned, and transformed using the TOPO®II TA Cloning® Kit (Invitrogen). Positive clones were confirmed by sequencing. For RNA/protein interaction studies,
sense transcripts, representing the 5′- or 3′-UTR of C-P4H-α(l) mRNA were prepared as described above and transcribed using the T7-polymerase. In vitro transcripts were purified by BD Chroma Spin™-100 (DEPC) columns (Clontech).

UV Cross-linking Experiments

In vitro transcripts representing the 5′- or 3′-UTRs of C-P4H-α(l) mRNA were radioactively labeled using [α-32P]uridine-, [α-32P]cytosine-, [α-32P]adenine-, or [α-32P]guanosine 5′-triphosphate (800 Ci/mmol, MP Biomedicals Germany GmbH).

UV Cross-linking Experiments

1–2 ng representing 100,000 cpm of [α-32P]UTP-labeled in vitro transcripts were incubated with 35 μg of cytosolic protein extract for 30 min at room temperature in 10 mM Hepes, pH 7.2, 3 mM MgCl2, 5% glycerol, 1 mM dithiothreitol, 150 mM KCl, and 2 units/μl RNase OUT (Invitrogen) in the presence of rabbit rRNA (0.5 μg/μl). Then the samples were exposed to UV light (255 nm, 1.6 joule, UV Stratalinker) on ice, treated with RNase for 30 min at room temperature in 10 mM Hepes, pH 7.2, 3 mM MgCl2, 5% glycerol, 1 mM dithiothreitol, 150 mM KCl, and 2 units/μl RNase OUT (Invitrogen) in the presence of rabbit rRNA (0.5 μg/μl). Then the samples were exposed to UV light (255 nm, 1.6 joule, UV Stratalinker). To map the protein-related qualitative and quantitative composition of each nucleotide in transcripts to nucleolin. The signal intensity depend on the experimental design, i.e. in vitro transcripts were similar in size and sequence. Hence, the influence of neighboring sequences as well as secondary structure are considered in the assay.

Affinity Chromatography

For the isolation of mRNA-binding proteins, in vitro transcripts representing the 5′- or 3′-UTR of C-P4H-α(l) mRNA were generated in the presence of biotinylated CTP (Invitrogen). Cytosolic extracts (5 mg protein) were incubated with 1 μg in vitro labeled transcript for 30 min at room temperature. RNP (ribonucleoprotein) complexes were isolated using 200 μl of streptavidin-agarose/sample (Sigma). Samples without the addition of biotinylated transcripts served as negative control. The agarose beads were centrifuged for 15 s at 5,000 × g and washed six times (20 mM Tris, pH 7.4, 150 mM KCl, 3 mM MgCl2, 0.5 mM dithiothreitol). The last two washing supernatants were used as control. The RNP complexes were eluted using high salt buffer (20 mM Tris, pH 7.4, 2 mM KCl, 3 mM MgCl2, 0.5 mM dithiothreitol). Proteins were precipitated, solved, and subjected to SDS-PAGE. After Coomassie staining protein signals representing specific RNA-binding factors were excised. Tryptic digestion of proteins was carried out using ZipPlates (Millipore) without reduction or alklylation. Tryptic fragments were analyzed by Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics). Mass spectra were analyzed using Mascot software 2.0 with automatic searches in NCBI nonredundant databases. Search parameters allowed for one misscleavage and oxidation of methionine. Criteria for positive identification of proteins with MS were set according to the scoring algorithm delineated in Mascot (28).

Mapping the Nucleolin Binding Motif in C-P4H-α(l) 5′-UTR using a Mathematical Approach

Relative amounts, r(A,C,G,U)p of UV cross-linking signals, corresponding to ~64-kDa nucleolin, were scanned and statistically evaluated. The results signal from the label transfer of separate radioactively labeled [α-32P]uridine-, [α-32P]cytosine-, [α-32P]adenine-, or [α-32P]guanosine 5′-triphosphate in vitro transcripts to nucleolin. The signal intensity depend on the quantitative and qualitative composition of each nucleotide in the RNA/protein interaction site. To map the protein-related intensity pattern of the relative amount, r(A,C,G,U)p of the cross-linking experiments to a sequence motif in the 5′-UTR of the P4H-α(l) mRNA the following algorithm was applied. A sliding window of 6 nt was shifted over the 5′-UTR. For each position, p, the relative theoretical amount a(A,C,G,U)p of each nucleotide in the window was determined.

Our mapping score is the inverse of an error function between the theoretical and the measured value within a sliding window. The error function calculates the sum of the squared differences of the theoretical and measured nucleotide fraction. At each position p the mapping score was determined according the following equation.

\[ ms(p) = \frac{1}{(r_a - a_{Ap})^2 + (r_c - a_{Cp})^2 + (r_g - a_{Gp})^2 + (r_u - a_{Up})^2} \]  

(Eq. 1)

A high value of ms(p) corresponds to a high probability of the motif matching the quantified radioactively labeled pattern. Possible effects of the neighborhood are considered by the experimental design, i.e. in vitro transcripts were similar in size and sequence. Hence, the influence of neighboring sequences as well as secondary structure are considered in the assay.
Results

Post-transcriptional Regulation of C-P4H-α(I) Expression under Long Term Hypoxia—Cell culture experiments, using human fibrosarcoma HT1080 cells, clearly demonstrate that C-P4H-α(I) is induced at the mRNA and protein levels under hypoxia (1% oxygen) (Fig. 1). Interestingly, during the late phase of a time scale up to 36 h C-P4H-α(I) protein increases independently of the mRNA concentration, suggesting a post-transcriptional component in the mechanism of expression control. We observed ~2-fold elevated mRNA and protein levels after 10 h hypoxia, compared with control. Whereas the mRNA concentration remained relatively constant also under long term hypoxic conditions (up to 36 h) and even dropped slightly, the protein level increased continuously. Under long term conditions C-P4H-α(I) protein levels increased nearly 6-fold, compared with a less than 3-fold increase at the mRNA level. The elevated mRNA level can be attributed to the transcriptional action of HIF (11). Consistently, we did not observe significant alterations of the C-P4H-α(I) mRNA stability (Fig. 2A and B). The significantly stronger increase seen at the protein level may have two reasons: either protein degradation was inhibited or translational efficiency was increased. Inhibition of translation by cycloheximide prevented the increase of the protein level by hypoxia, indicating that the elevated protein concentration was due to newly synthesized protein (Fig. 2C and D). Furthermore, the C-P4H-α(I) protein level dropped to about one-third by cycloheximide under both, hypoxic and atmospheric conditions. This suggests that the rate of protein degradation was not affected by hypoxia.

To determine whether C-P4H-α(I) is regulated at the posttranscriptional level, we generated reporter gene constructs. For this purpose 5'- and 3'-UTRs of luciferase mRNA were replaced by specific 5'- and/or 3'-UTRs of C-P4H-α(I) mRNA (for a schematic illustration see Fig. 3A), and reporter gene assays were performed after transient transfection. The transcription rate of the reporter gene was controlled by a constitutive SV40 promoter. Thus, differences in luciferase activity depended solely on the regulatory capacity mediated by the C-P4H-α(I) UTRs. Long term hypoxia (36 h) did not influence the luciferase expression/activity resulting from the original reporter gene. The replacement of the original 5'-UTR by the C-P4H-α(I) 5'-UTR also showed no significant changes, whereas the replacement of the 3'-UTR led to a 1.3-fold increased luciferase activity. Interestingly, the combination of C-P4H-α(I) 5'- and 3'-UTRs potentiated the effect: expression reached a 2.2-fold level versus control (Fig. 3B), which correlated well with the discrepancy between mRNA and the protein level under long term hypoxia. Dividing the 3'-UTR into two parts (termed 3'A and 3'B) did not result in a comparable activation (Fig. 3C). Neither the individual 5' ~500 nt, nor the terminal 3' ~500 nt of the 3'-UTR reached the full activity of the complete 3'-UTR. Obviously, 3'-UTR parts did not represent the properties of the complete 3'-UTR. This is in contrast to findings after Fe^{2+} diminishment, where the regulative properties of the 3'-UTR part B is dominated by a U-rich element (27).

**Statistical Analysis**

Autoradiographic signals were scanned and quantified using the Scion Image software (Scion Corp.). Results appear as means, and error bars represent the standard deviation (S.D.). Data were analyzed using the Student’s t test, and the null hypothesis was rejected at the 0.05 level.

**RESULTS**

**Post-transcriptional Regulation of C-P4H-α(I) mRNA and Protein in response to hypoxia (1% oxygen).** Human fibrosarcoma cells (HT1080) were cultivated up to 36 h under control (C) or hypoxic (Hy) conditions. A, C-P4H-α(I) mRNA levels were determined by RT-PCR, protein levels by Western blotting. β-Actin served as a control. Shown are means of six independent experiments. B, statistical analyses. Relative values are normalized to β-actin, the numerical values are means, error bars represent the standard deviation (n = 6).

**FIGURE 1.**
The complete 3′-UTR showed a weak independent influence (1.3-fold), the 5′/3′-UTR combination seems to be a prerequisite for an optimal translational efficiency of C-P4H-α(I) mRNA under hypoxia.

The data revealed that under long term hypoxia C-P4H-α(I) in HT1080 fibroblasts is not only regulated at the transcriptional level (11), but is dependent on a 5′/3′-UTR interaction in a posttranscriptional process. We did not observe a significant influence on mRNA stability, and conclude that the posttranscriptional regulation is mainly attributed to the modulation of translational efficiency through 5′/3′-UTR interaction.

C-P4H-α(I) UTR/Protein Interaction—Posttranscriptional regulation is mainly established through RNA/protein interaction. We used an avidin/biotin-based affinity chromatography approach (13, 29) to isolate C-P4H-α(I) 5′- and 3′-UTR-binding proteins as possible candidates for factors involved in posttranscriptional regulation of C-P4H-α(I) expression. The results of protein identification by MALDI-TOF-MS analysis are shown in Table 1. Nucleolin, ribosomal protein L7a, and eukaryotic translation elongation factor α1 were identified to interact at the 5′-UTR. Nucleolin was also found to participate in the 3′-UTR RNP assembling, as well as the splicing factor proline/glutamine-rich, heat shock 70-kDa protein 8 isoform 2, the members of hnRNP family hnRNP-R, hnRNP-A2/B1, and hnRNP-A3, annexin A2, and BRD3 protein. All identified proteins are known RNA-binding proteins and potential mediators of posttranscriptional control in C-P4H-α(I) gene expression. However, these findings have to be verified by further investigations. Earlier hnRNP-A2/B1 was shown by us to interact with an U-rich element within the 3′-UTR and modulates C-P4H-α(I) mRNA stability (27).

As a next step, we performed UV cross-linking assays to detect changes in the binding behavior of trans-factors (RNA-binding proteins) in response to hypoxia. The data indicate multiple quantitative changes in the binding properties of 5′- as well as 3′-UTR-binding proteins (Fig. 4). The most prominent alteration of a UV cross-linking signal was observed at the 5′-UTR. It corresponded to a ~64-kDa RNA-binding protein. From the data obtained from UTR-dependent reporter gene assays it was evident that the 5′-UTR did not promote a posttranscriptional control on its own. This result supported the view that the interaction of the ~64-kDa protein with the
5′/3′-UTR Interaction of C-P4H-α(I) mRNA

**A**

*pGL3p* [SV40] Luciferase

5′UTR-Luc [SV40] 5′UTR Luciferase

Luc-3′UTR [SV40] 3′UTR Luciferase

5′UTR-Luc-3′UTR [SV40] 5′UTR (A) 3′UTR (B)

Luc-3′A [SV40] 3′A

Luc-3′B [SV40] 3′B

5′UTR-Luc-3′B [SV40] 5′UTR Luciferase 3′B

**B**

Luc-3′A

Luc-3′B

5′UTR-Luc-3′A

5′UTR-Luc-3′B

**C**

**FIGURE 3. Influence of C-P4H-α(I) mRNA UTRs on luciferase expression.** HT1080 cells were transfected using the *pGL3*-promoter vector (SV40 promoter) and transgenic variants, in which vector-specific 5′- and 3′-UTRs of luciferase mRNA were replaced by C-P4H-α(I) UTRs. The influence of hypoxia (1% oxygen, 36 h) on luciferase activity of the original vector (*pGL3p*) and vector constructs with C-P4H-α(I) 5′- and/or 3′-UTR sequences are shown as black bars. A, schematic illustration of reporter gene constructs. The dotted lines represent the coding sequence of luciferase transcript. Boxes termed Luciferase represent the coding sequence of luciferase transcript. B, statistical analyses of results regarding original luciferase transcript and native C-P4H-α(I) UTRs (n = 12, *, p < 0.05; **, p < 0.01). Shown are relative values compared to control levels. The combination of C-P4H-α(I) 5′- and 3′-UTR significantly enhances the UTR-mediated luciferase activity under hypoxia, compared with the hypoxic response of separate 5′- or 3′-UTRs. C, impact of hypoxia (36 h) mediated 5′/3′-UTR interaction on luciferase activity by combination of C-P4H-α(I) 5′-UTR with artificial 3′-UTR parts. 3′A represent the 5′ localized 500 nt and 3′B represent the 3′ terminal 522 nt of the complete 3′-UTR as described in Ref. 27. Shown are relative values. The results show that 3′-UTR parts do not mediate a 5′/3′-UTR cross-talk, and therefore do not reflect the feature of the complete 3′-UTR.

5′-UTR is involved in a functional 5′/3′-UTR cross-talk. We therefore focused our further efforts on this ~64-kDa protein.

MALDI-TOF-MS analysis identified this ~64-kDa protein as nucleolin. Hypoxia, however, did not change the nucleolin mRNA level (Fig. 5A). Western blotting analyses revealed that nucleolin may be regulated at the posttranslational level. We observed a marked decrease of a ~100-kDa nucleolin form in favor of an increase in smaller nucleolin fragments (Fig. 5B). The increase of a ~64-kDa nucleolin form, which shows mRNA binding properties, could explain the increased binding to the 5′-UTR. Interestingly, this particular nucleolin form was also identified as 3′-UTR-binding protein by RNA affinity chromatography, but we did not observe a distinct nucleolin-related UV cross-linking signal in several independent experiments. UV cross-linking signals represent proteins, which bind to the RNA by direct RNA/protein interaction, so we conclude that the interaction of nucleolin at the C-P4H-α(I) 3′-UTR was brought about by a secondary protein/protein interaction between nucleolin and other RNA-binding proteins. One of these possible nucleolin interacting proteins is annexin A2, which was experimentally identified as C-P4H-α(I) 3′-UTR-binding protein (see Table 1). To test our line of reasoning we performed differential centrifugation assays, investigating separately a translationally active fraction (1,000–100,000 × g), which includes rough endoplasmic reticulum residues, free polysomes and other polysomes-associated aggregates, and a translationally inactive (100,000 × g supernatant) polysomes-free fraction, containing ribonucleoprotein complexes, as well as free RNAs and cytosolic proteins under hypoxia. We observed a nearly 5-fold increase of C-P4H-α(I) mRNA in the polysomal fraction (Fig. 6A). This increase seen in the translationally active fraction exceeds the overall induced mRNA.
level (which is ~2.5-fold), and can be explained by a significant decrease of C-P4H-α(I) mRNA concentration in the translationally inactive RNP fraction. These findings indicate a recruitment of C-P4H-α(I) mRNAs into polysomes, which supports the view of an enhanced translational control under hypoxia. Furthermore, the C-P4H-α(I) mRNA-binding proteins nucleolin (~64 kDa form) (Fig. 6B) and annexin A2 (Fig. 6C) are enriched in the polysomal fraction as a result of hypoxia, which is in line with the finding regarding its bound C-P4H-α(I) mRNA. Interestingly, not all nucleolin forms shifted into the translationally active fraction. We observed an elevated presence of the high molecular mass forms (~100 kDa) as well as the ~64-kDa nucleolin fragment in the polysomal fraction, whereas smaller nucleolin fragments are located predominantly in the postpolysomal fraction (Fig. 6B). In summary, UV cross-linking and affinity chromatography revealed that the ~64-kDa nucleolin form bound directly to the 5′-UTR and interacted indirectly with the 3′-UTR, causing an elevated recruitment of ribosomes, and consequently, an increased translational efficiency.

Identification of ~64-kDa Nucleolin/C-P4H-α(I) 5′-UTR Interaction Site—To analyze the cis-element (RNA-binding motif) of the C-P4H-α(I) mRNA 5′-UTR in detail, which is responsible for nucleolin interaction, we carried out competition assays. Different parts of the 5′-UTR were transcribed in vitro and added in 50-fold molar excess to the UV cross-linking samples (Fig. 7A). The competition assay revealed that the ~64-kDa nucleolin signal could not be suppressed by the 5′-UTR 1–110 nt of the 5′-UTR. However, transcripts representing the 3′-terminal 23 nt of the 5′-UTR effectively suppressed it. Central to this 23-nt region is an UAAAAUUAAAU motif (see Fig. 9A). It seems to be involved in binding of several trans-factors. However, under hypoxic conditions the increased binding capacity of the ~64-kDa nucleolin is the most obvious alter-

### TABLE 1

| RNA-binding proteins, interacting with the 5′- and 3′-UTR of C-P4H-α(I) mRNA |
|-------------------------------------------------------------|
| RNA-binding proteins were purified by affinity chromatography using biotinylated in vitro transcripts, which represent the 5′- or 3′-UTR of P4H-α(I) mRNA. Proteins were identified by MALDI-TOF-MS analysis. |

| 5′-UTR                      | 3′-UTR                      |
|-----------------------------|-----------------------------|
| Nucleolin (~64 kDa), gi:4885511 | Nucleolin (~64 kDa), gi:4885511 |
| Ribosomal protein L7a, gi:4506661 | Splicing factor proline/glutamine-rich, gi:826998 |
| Eukaryotic translation elongation factor 1a, gi:4503471 | HSP-A8, gi:24234686 |
|                             | hnRNP-R, gi:13629286 |
|                             | hnRNP-A2/B1, gi:14043072 |
|                             | hnRNP-A3, gi:34740329 |
|                             | Annexin A2, gi:16309678 |
|                             | BRD3 protein (syn. ORFX, RING3L), gi:21594670 |

![FIGURE 4. Influence of hypoxia on interaction of cytosolic proteins with C-P4H-α(I) mRNA UTRs, analyzed by UV cross-linking.](image)

**FIGURE 4.** Influence of hypoxia on interaction of cytosolic proteins with C-P4H-α(I) mRNA UTRs, analyzed by UV cross-linking. [32P]UTP-labeled in vitro transcripts, which represent the 5′- or 3′-UTR of C-P4H-α(I) mRNA, were incubated with cytosolic extracts, isolated from cells exposed to control (C) or hypoxic (Hy) conditions. The arrowhead marks a ~64-kDa protein (nucleolin), which shows an increased binding capacity in response to hypoxia.

![TABLE 1](image)

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![FIGURE 5. Formation of nucleolin subfragments by hypoxia.](image)

**FIGURE 5.** Formation of nucleolin subfragments by hypoxia. Human fibrosarcoma cells (HT1080) were cultivated up to 36 h under control or hypoxic conditions. A, RT-PCR was performed to detect relative mRNA levels. Hypoxia did not influence the nucleolin mRNA concentration. B, Western blotting analysis shows relative nucleolin protein levels. A pool of six independent samples is shown. β-Actin served as loading control. The formation of the ~64-kDa nucleolin, active in translational control, is increased as a result of hypoxia.
**5′/3′-UTR Interaction of C-P4H-α(l) mRNA**

| total RNA | polysomal fraction | RNP fraction |
|-----------|-------------------|--------------|
| control   | control            | control      |
| 1 ± 0.20  | 4.79 ± 0.69        | 1 ± 0.32     |
| hypoxia   | hypoxia            | hypoxia      |
| 2.52 ± 0.25| 0.18 ± 0.69       | 0.32 ± 0.11  |

< C-P4H-alpha (I)

< 28S rRNA < 18S rRNA

**FIGURE 6. Localization of C-P4H-α(l) mRNA, as well as nucleolin and annexin A2 proteins in translational active or inactive fractions.** HT1080 cells were cultivated under control or hypoxic conditions for 24 h. Cellular extracts were separated by ultracentrifugation in a polysomal fraction (1,000–100,000 × g), which contained rough endoplasmic reticulum residues, free polysomes, and other polysomal aggregates, and a 100,000 × g supernatant (RNP fraction), which contained translationally inactive RNP (1,000–100,000 × g) extracts were separated by ultracentrifugation in a polysomal fraction. A, representative Northern blotting analysis of the C-P4H-α(l) mRNA level. Three independent samples were statistically evaluated. The hypoxic induction of the C-P4H-α(l) mRNA level under these methodical conditions (total RNA) served as a comparison, and is in line with the data obtained by RT-PCR. The C-P4H-α(l) mRNA level in the translationally inactive (RNP) fraction is decreased in favor of an increase in the polysomal fraction. B and C, Western blotting analysis to detect the localization in RNA/protein interaction and may refer to a posttranscriptional effect on gene expression. To confirm the AU-rich element/nucleolin interaction, we performed additional UV cross-linking assays. UV cross-linking signals, corresponding to RNA-binding proteins, depend on the direct interaction of trans-acting factors with cis-acting elements. The observed signal intensity depends further on the quality and quantity of nucleotides, which are involved in the RNA/protein interaction (30). The separate labeling of 5′-UTR transcripts with [α-32P]-UTP, -CTP, -ATP, or -GTP revealed that the nucleolin binding site is indeed AU-rich (Fig. 7B). To compare RNA-binding properties of 3′-UTR-binding proteins we also labeled transcripts representing the 3′-UTR separately (Fig. 7C). We observed no ~64-kDa UV cross-linking signal at the 3′-UTR with similar binding properties seen by nucleolin at the 5′-UTR, which supports independently the view that the mechanism of nucleolin interaction differs between 5′- and 3′-UTRs.

For fine-mapping of the C-P4H-α(l) 5′-UTR/nucleolin interaction site we used a new mathematical strategy, based on the relative abundance of nucleotides obtained by UV cross-linking assays. A high score value was observed for sequences UAAUC and AAUAC (Fig. 8), which correlated well with the data obtained by competition assays. The data, furthermore, indicate that nucleolin interacts only with parts of the AU-rich element.

To confirm the functional importance of the C-P4H-α(l) 5′-UTR/nucleolin interaction in the modulation of translational efficiency of C-P4H-α(l) mRNA under hypoxia, we mutated the identified cis-element, as well as the flanking regions and performed additional UTR-dependent reporter gene assays. The results show that not only the mutation of the calculated nucleolin interaction site, but also mutations of the flanking regions reduced the hypoxia inducible luciferase activity (Fig. 9A). 5′-UTR mutations did not abolish the UTR-mediated response to hypoxia completely, which can be attributed to the independent influence of the 3′-UTR (see Fig. 3B). Furthermore, mutations of the hypoxia response relevant part of the C-P4H-α-5′-UTR caused a qualitative change in RNP assembling, seen by UV cross-linking assays (Fig. 9B). This variation included a loss of the binding ability of nucleolin, important for the 5′/3′-UTR cross-talk-mediated hypoxic response. The results further support the finding that the 3′ terminal part of the 5′-UTR represents an important interaction site, not only for nucleolin. The functional significance of this region is supported by the observation that the mutations led to a lower gene expression rate, compared with the non-mutated C-P4H-α(l) 5′-UTR (Fig. 9C). This finally demonstrates a crucial role of the 5′-UTR in the modulation of C-P4H-α(l) translational efficiency, not only under hypoxia.
Hypoxia is important under variable physiological conditions (e.g. altitude, hibernation, diving mammals, and working muscles) and in pathophysiological settings (ischemia, anemia, and diffusion barriers). Furthermore, hypoxia is a central issue in tumorigenesis. The first striking cellular response to hypoxia is the suppression of energy consuming processes, such as translation, protein degradation, and transcription (31, 32). Although the overall metabolic rate is suppressed, several genes show an increased expression rate against the trend, which has been attributed to activation of the transcription factor HIF. The alteration of gene expression can be modulated further at the posttranscriptional or posttranslational levels. Posttranscriptional control involves alteration in mRNA stability, translational efficiency, or processes related to mRNA localization. In particular, mRNA translation is a highly controlled process that is sensitive to a variety of cellular stressors (22–24). Hitherto, the hypoxic adaptation of mRNA translation was attributed to the phosphorylation of the essential eukaryotic initiation factor eIF2α, or mTOR-mediated inactivation of eIF4F (see Ref. 33).

The induction of fibrosis following hypoxia is of major importance. The enhanced expression and deposition of collagen under hypoxia appears paradoxical, due to the formation of diffusion barriers, which further impair oxygen supply. Obviously the hypoxic-induced fibrosis is a conserved pathway and serves the evolutionary importance of wound healing and scarring, because hypoxia constitutes a local condition after wounding. The restoring of blood and oxygen supply may be attributed to HIF, a factor known to induce angiogenesis.

The increased collagen synthesis requires posttranslational modifications, especially the hydroxylation by C-P4H. The C-P4H activity depends on oxygen, and decreased oxygen levels seem to be compensated through an enhanced expression of the limiting C-P4H-α subunit. Hitherto, the elevated expression of C-P4H-α(I) was mainly attributed to the transcriptional regulation by HIF under hypoxic conditions (11). We observed in human fibroblasts (HT1080 fibrosarcoma cells) that, compared with control conditions, under long term hypoxia (36 h) the C-P4H-α(I) protein level was induced to a greater extent than its mRNA level. Cycloheximide, an inhibitor of transla-

**FIGURE 7. Characterization of C-P4H-α(I) 5′-UTR/nucleolin interaction.** 32P-Labeled in vitro transcripts, which represent the 5′-UTR of C-P4H-α(I) mRNA, were incubated with cytosolic extracts. A, mapping of C-P4H-α(I) 5′-UTR/protein interaction by competition. Nonlabeled in vitro transcripts, representing partial 5′-UTR sequences, were added in an ~50-fold molar excess to the radioactively labeled (*) 5′-UTR (133 nt in size) as competitor. The suppression of signals, corresponding to 5′-UTR-binding proteins, indicate RNA/protein interaction sites. An unspecified vector transcript served as control. B, free probe, B and C, label transfer to RNA-binding proteins of C-P4H-α(I) 5′-UTR or 3′-UTR (C) using [α-32P]uridine-, [α-32P]cytosine-, [α-32P]adenine-, or [α-32P]guanosine 5′-triphosphate-labeled transcripts was performed by the UV cross-linking technique. The signal intensity indicates the qualitative and quantitative involvement of each nucleotide in the RNA/protein interaction. The separate labeling of C-P4H-α(I) 3′-UTR is shown as a comparison.

**FIGURE 8. Mapping of the motif position of the ~64-kDa nucleolin interaction site in the 5′-UTR of C-P4H-α(I) mRNA.** Relative amounts rAACCU of UV cross-linking signals at the molecular mass of nucleolin (~64 kDa) were statistically evaluated (n = 4), resulting in an experimentally obtained nucleotide abundance (inset) of the nucleolin binding motif in UV cross-linking assays (see Fig. 7B). A sliding window of 6 nt was shifted over the 5′-UTR. The calculated mapping score is a function of the motif position. High values of the score correspond to a good match with the obtained nucleotide abundance. The results show that the nucleolin interaction site fits best either to UAAUUC or AAAAAUC.

**DISCUSSION**

Hypoxia is important under variable physiological conditions (e.g. altitude, hibernation, diving mammals, and working muscles) and in pathophysiological settings (ischemia, anemia, and diffusion barriers). Furthermore, hypoxia is a central issue in tumorigenesis. The first striking cellular response to hypoxia is the suppression of energy consuming processes, such as translation, protein degradation, and transcription (31, 32).
tion, suppressed the increased C-P4H-α(I) protein level under hypoxia, supporting the view that the increased protein concentration required the synthesis of new protein. Furthermore, a 24-h cycloheximide treatment decreased the C-P4H-α(I) protein level to one-third under both control and hypoxic conditions. This observation indicates that the rate of protein decay is similar in both settings. The results show that C-P4H-α(I) expression is controlled by transcriptional as well as by post-transcriptional mechanisms under conditions of a depression of the metabolic rate.

C-P4H-α(I) UTR-dependent reporter gene assays revealed that with respect to long term hypoxia, the presence of both 5'- and 3'-UTR is primarily important in the posttranscriptional control. The requirement of the 5'- and 3'-UTRs in the posttranscriptional control of C-P4H-α(I) expression may be explained by the “closed loop mRNA” model, which is commonly associated with an enhanced translational efficiency (34). Hence, we observed a clearly increased presence of the C-P4H-α(I) mRNA, as well as its bound proteins in the polysomal fraction. The 5'/3'-UTR cross-talk is not observed by artificial 3'-UTR parts, and only the complete 3'-UTR supports the posttranscriptionally mediated hypoxic response. Using different techniques we identified nucleolin (∼64 kDa form) as a crucial factor providing a link between the 5'- and 3'-UTRs. Nucleolin, expressed as a ∼100-kDa protein, is among the most abundant non-ribosomal proteins of the nucleolus and is important in ribosomal biogenesis, which involves transcription and processing of pre-rRNA, as well as nucleocytoplasmic transport (35). Furthermore, nucleolin interacts with ribosomal proteins and transcription factor complexes (36–38), as well as with small RNP s (39). Nucleolin is also important in nuclear translocation of S100/A11 in Ca²⁺-induced growth inhibition (40). Despite its nuclear function, nucleolin has been shown to be a marker at the cell surface of angiogenic endothelial cells.
(41), and also plays an important role in the cytoplasm (42). Cytoplasmic nucleolin was regarded as an mRNA stabilization factor (29, 43–45) and is involved in translational control (46–48). Interestingly, nucleolin can undergo an autocatalytic cleavage into distinct fragments (49). Singh et al. (45) showed at least 8 different nucleolin forms, which may have different properties and functions. In the present work we demonstrate that a ~64-kDa nucleolin fragment has mRNA-binding properties and is associated with an enhanced translational efficiency of the C-P4H-α(I) mRNA. Recently we showed that in the absence of Fe^{2+} ions, this ~64-kDa nucleolin form binds increasingly at the 3′-UTR of matrix metalloproteinase-9 mRNA, and causes an enhancement in translation (48). Fe^{2+} depletion also affects the posttranscriptional control of C-P4H-α(I) expression by the nucleolin-mediated 5′/3′-UTR interaction. In contrast to hypoxia, treatment by an iron chelator also affects the 5′-UTR-binding properties of other proteins, resulting in less importance of nucleolin mediated control up to 18 h (27). However, under hypoxia (36 h) the ~64-kDa nucleolin-mediated transnational control is the dominant posttranscriptional influence controlling C-P4H-α(I) synthesis. Nucleolin binds directly at the C-P4H-α(I) 5′-UTR and, probably mediated through other RNA-binding proteins, indirectly at the 3′-UTR. The identification of C-P4H-α(I) 3′-UTR-binding proteins revealed that annexin A2, an already known nucleolin-binding protein (50), participates in 3′-UTR/protein assembling. The constitutive presence of this or other 3′-UTR-binding proteins may be crucial for the 5′/3′-UTR interaction, because they present a link to the 5′-UTR by nucleolin interaction.

The RNA binding property of the distinct ~64-kDa nucleolin fragment is different to the known rRNA-related binding element (units/G)CCCG(A/G) (51, 52) of the high molecular weight form. The C-P4H-α(I) 5′-UTR recognition motif, interacting with nucleolin, involves an AU-rich element and corresponds to UAAAUUC or AAAUCU. We calculated this core sequence for nucleolin binding using a new mathematical approach, based on the experimentally confirmed qualitative and quantitative involvement of each possible nucleotide in the nucleolin binding motif by UV cross-linking assays. As mentioned above we recently also identified this ~64-kDa nucleolin form as RNA-binding protein, interacting with the 3′-UTR of matrix metalloproteinase-9 mRNA. We favor the calculated UAAAUUC sequence as a core binding motif for ~64-kDa nucleolin, because this motif is also present in the 3′-UTR of matrix metalloproteinase-9 mRNA. However, functional UTR-dependent reporter gene assays revealed that not only the direct interaction motif is responsible for the nucleolin interaction. Mutations of the flanking regions also inhibit the 5′/3′-UTR cross-talk-mediated hypoxic response and nucleolin binding seen in UV cross-linking assays. These observations may be explained by the importance of mRNA secondary structures and/or other proteins in the recruitment of RNA-binding factors and assembling of RNP complexes. Consistently, nucleolin was described to optimize access of other RNA-binding proteins to its functional binding sites (53). The involvement of an AU-rich element in the RNA/nucleolin interaction was previously described by Sengupta et al. (54), who showed that ~100- and ~70-kDa nucleolin forms affect the mRNA stability by interacting with a classical ARE within the 3′-UTR of bcl-2 mRNA. These findings support the view that nucleolin and its distinct fragments are multifunctional and influence gene expression at different levels.

Furthermore, we observed that the 3′ terminal part of the C-P4H-α 5′-UTR, including the AU-rich element, also affects the binding properties of several other 5′-UTR-binding proteins shown by competition and UV cross-linking assays. Mutation of this region led to a qualitative change of binding properties of 5′-UTR interacting proteins and decreased the basal expression rate in UTR-dependent reporter gene assays. Under hypoxic conditions the absence of nucleolin interaction appears crucial, and caused a loss in the ability of the 5′/3′-UTR interaction-mediated elevated expression rate. The results clearly show the importance of the 5′-UTR in the modulation of C-P4H-α(I) gene expression, which was postulated earlier (55).

Apart from nucleolin, we identified other putative regulatory factors interacting with the C-P4H-α(I) UTRs by MALDI-TOF-MS analysis. All identified proteins (see Table 1) represent known RNA-binding factors and may be necessary in the posttranscriptional regulation of C-P4H-α(I) expression under different environmental conditions, in different developmental stages or cell types. UV cross-linking assays indicate that more UTR-binding proteins are yet to be identified. In this study, we focused on the RNA-binding factor nucleolin, because under hypoxic conditions the nucleolin mediated influence in the posttranscriptional alteration of C-P4H-α(I) expression appeared dominant. The importance of the other candidates has to be confirmed by additional experiments under different physiological conditions.

Finally, we hypothesize that the posttranslational regulation of nucleolin by autocatalytic cleavage into distinct fragments may be a key step in the enhanced translation rate of a set of specific mRNAs under hypoxia. We observed no alteration at the nucleolin mRNA level following hypoxia, indicating a HIF independent response. Nucleolin was shown to be a Ca^{2+}-binding protein (56). Hypoxia is a stimulus that modulates Ca^{2+} signaling, mediated by voltage-gated calcium entry through oxygen-sensitive potassium channels (57–59), calcium-sensitive potassium (BK) channels regulated by hemoxynase-2 (60), as well as liberation of Ca^{2+} from the endoplasmic reticulum through regulation of reactive oxygen species and via ryanodine receptors (61). The HIF independent response at the level of translation may therefore be linked to the Ca^{2+} signaling, affecting posttranslational control of nucleolin and translational efficiency of certain mRNAs. Recently, the ~100-kDa form of nucleolin was identified as a putative translational repressor of p53 mRNA translation (47). However, the dramatic decrease of this high molecular weight nucleolin form in favor of distinct fragments as a result of hypoxia, may cause a switch from translational inhibition to an enhanced rate of translation of a specific set of mRNAs. Furthermore, the multiple functions of nucleolin, as mentioned above, may affect the metabolic regulation under hypoxia in a broad manner, including ribosomal biogenesis, RNA/protein shuttling, RNP configura-
5′/3′-UTR Interaction of C-P4H-α(I) mRNA

tions, protein/protein interaction, as well as translational control. We hypothesize that nucleolin may participate in the recruitment of specific mRNA transcripts into stress granules, an important step in the stress-induced alteration of mRNA stability and translational properties (reviewed in Ref. 62).

In summary, enhanced C-P4H-α(I) expression is not only regulated at the transcriptional level in response to hypoxia. Under conditions where energy-consuming processes are suppressed, the synthesis of C-P4H-α(I) protein is enhanced by posttranscriptional control. The increase in the concentration of the limiting α(I) subunit (necessary to form an active C-P4H tetramer) can partially be attributed to a 5′/3′-UTR interaction, leading to an enhanced translational efficiency under long term hypoxia. The posttranscriptional control of C-P4H-α(I) expression is strongly associated with the increased binding of a ~64-kDa nucleolin form at the 5′- as well as 3′-UTR. The interaction of nucleolin appears to be attributed to the 3′ terminal part of the 5′-UTR, and involves an UAAUC motif as the direct contact site. Interaction of nucleolin at the 3′-UTR requires additional RNA-binding factors, possibly annexin A2.

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