Increased type I collagen content and DNA binding activity of a single-stranded, cytosine-rich sequence in the high-salt buffer protein extract of the copper-deficient rat heart

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Abstract

Dietary copper (Cu) deficiency not only causes a hypertrophic cardiomyopathy but also increases cancer risk in rodent models. However, a possible alteration in gene expression has not been fully examined. The present study was undertaken to determine the effect of Cu deficiency on protein profiles in rat heart tissue. Male Sprague-Dawley rats were fed diets that were either a Cu-adequate diet (6.0 μg Cu/g diet, $n = 6$) or a Cu-deficient diet (0.3 μg Cu/g diet, $n = 6$) for 5 weeks. The high-salt buffer (HSB) protein extract from heart tissue of Cu-deficient, but not Cu-adequate rats showed a 132 kDa protein band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. This protein band stained pink with Coomassie Blue, suggesting the presence of collagens or other proline-rich proteins. Dot immunoblotting demonstrated that total type I collagen was increased by 110% in HSB protein extract from Cu-deficient, relative to Cu-adequate, rats. Liquid chromatography with mass spectrometry analysis indicated that the 132 kDa protein band contained a collagen α (I) chain precursor as well as a leucine-rich protein 130 (LRP130) in HSB protein extract from Cu-deficient but not Cu-adequate rats. A gel shift assay showed that HSB protein extract from Cu-deficient rats bound to a single-stranded cytosine-rich DNA with higher affinity than the extract of Cu-adequate rats, similar to reports of an increase in LRP130 single-stranded DNA binding activity in several types of tumor cells. Collectively, these results not only suggest an additional feature of altered collagen metabolism with Cu deficiency but also demonstrate for the first time an increase in single-stranded cytosine-rich DNA binding in Cu-deficient rat heart. © 2004 Elsevier Inc. All rights reserved.

Keywords: Copper deficiency; Heart; Collagen; DNA binding

1. Introduction

Copper (Cu) is an essential transition metal required for the activity of multiple mammalian enzymes including Cu/Zn-superoxide dismutase, lysyl oxidase, and ceruloplasmin [1,2]. The diversity of functions and tissue expression of cuproenzymes suggest multiple roles for this essential nutrient. An inadequate intake of Cu restricts the activity of cuproenzymes, and Cu deficiency has been implicated in a number of conditions including cardiomyopathy, impaired immune function and higher cancer risk [3–5]. Dietary Cu deficiency in the growing rat results in a number of cardiovascular disorders such as concentric cardiac hypertrophy, which is a consequence of a variety of pathophysiological stimuli. This is thought to be due in part to a reduction in the activity of the cuproenzyme lysyl oxidase, which is required in collagen and elastin cross-linking in the extracellular cardiac matrix. It has been suggested that this reduced activity could alter the loading of the myocytes and lead to hypertrophy [6–8]. However, other studies have shown that treatment of rats with the lysyl oxidase inhibitor β-amino proprionitrile reduces myocardial tissue stiffness but does not alter heart size and ventricular wall fragility [9,10]. These physiological changes likely depend primarily on the decreased activity of cuproenzymes, but several studies

have demonstrated that cardiac hypertrophy observed in Cu-deficient male rats may involve the dysregulation of numerous genes such as reactivation of certain proto-oncogenes [11,12]. Cardiomyocytes are terminally differentiated and lose their ability to proliferate soon after birth. At the cellular level, cardiac hypertrophy is associated with an increase in cell size and protein synthesis. In view of the differential gene expression related to cardiac hypertrophy caused by Cu deficiency, it is logical to assume that cardiac transcription factors play a key role because they directly regulate a number of cardiac genes that are involved in cardiac hypertrophy [13–15]. A high-salt buffer extraction [16] is a well accepted procedure to enrich nuclear transcription factors even though the extracted fraction still contains other cellular proteins from other organelles such as mitochondria. In the present study, we used the high-salt buffer extraction method [16] to examine protein profiles of Cu-deficient and Cu-adequate rat hearts. Overall, these data may be useful in deciphering the change in gene expression related to dietary Cu deficiency.

2. Methods and materials

2.1. Chemicals

T4 polynucleotide kinase was obtained from Promega (Madison, WI). Adenosine 5′-triphosphate (γ-^{32}P) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotides were synthesized by Gibco BRL (Rockville, MD).

2.2. Animals and diets

Experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" [17] and were approved by the Animal Care Committee of the Grand Forks Human Nutrition Research Center.

Twelve male, 3-week old, weanling Sprague-Dawley rats (Charles River/Sasco, Wilmington, MA) were divided into two dietary groups. Diets were composed of 940.0 g of copper (Cu)–free, iron (Fe)–free basal diet (catalog no. TD 84469, Teklad Test Diets, Madison, WI), 50.0 g of safflower oil, and 10.0 g of Cu-Fe mineral mix per kilogram of diet. The basal diet was a diet based on casein (200 g/kg), sucrose (386 g/kg), and cornstarch (295 g/kg) and containing all known essential vitamins and minerals except Cu and Fe [18]. The mineral mix contained cornstarch and Fe with or without Cu, and provided 0.22 g of ferric citrate (16% Fe) and either 0 or 24 mg of added $CuSO_4 \cdot 5H_2O$ per kilogram of diet. These formulations were intended to provide a severely Cu-deficient diet (CuD) containing only Cu present in the basal diet and a Cu-adequate diet (CuA) containing 6 mg/kg of diet. Triplicate dietary analyses (see below) of each diet indicated average Cu concentrations of 0.28 and 6.20 mg of Cu/kg of diet for the CuD and CuA diets, respectively.

Analysis of dietary Cu was performed by dry ashing of the diet sample [19], dissolution in aqua regia and measurement by atomic absorption spectroscopy (model 503, Perkin-Elmer, Norwalk, CT). The assay method was validated by simultaneous assays of a wheat flour reference standard (National Institute of Standards and Technology, Gaithersburg, MD) and a dietary reference standard (HNRC-1A) that was developed by the Grand Forks Human Nutrition Research Center.

After the rats consumed their respective diets for 5 weeks, each rat was anesthetized with an intraperitoneal injection of thiobutabarbital sodium (Inactin, Research Biochemicals International, Natick, MA; 100 mg/kg body weight). Blood was withdrawn from the inferior vena cava into test tubes treated with ethylene diamine tetraacetate (EDTA), and hemoglobin and hematocrit were determined with a cell counter (Cell-Dyn, model 3500CS, Abbott Diagnostics, Santa Clara, CA). The median lobe of the liver was excised for mineral assays. Liver Cu and Fe concentrations were determined by lyophilizing and digesting organ samples with nitric acid and hydrogen peroxide [20] and measuring Cu concentration by inductively coupled argon plasma emission spectroscopy (Model 1140, Jarrell-Ash, Waltham, MA).

Hearts were excised and placed in phosphate-buffered saline on ice for subsequent protein extraction, as described below.

2.3. Preparation of high-salt buffer protein extract

Unless otherwise indicated, all operations were performed at 4°C. High-salt buffer (HSB) protein extracts were prepared by modifying a generally accepted procedure [16]. Fresh tissues from heart muscle were finely minced in phosphate-buffered saline and centrifuged at $532 \times g$ for 5 minutes. The pellets were lysed in lysis buffer (20 mmol/L HEPES, pH 7.6, 20% glycerol, 0.5 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and leupeptin, 10 mg/L) in a Wheaton Dounce homogenizer. Nuclei and other organelles were collected by centrifugation at $532 \times g$ for 5 minutes, suspended in lysis buffer containing 500 mmol/L NaCl, gently rocked for 1 hour, and then centrifuged at $15,000 \times g$ for 15 minutes. The supernatant was designated the HSB protein extract and kept at -80°C.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS) gel system with gels 1.5 mm in thickness was used [21]. Usually, 50 μg of HSB protein extract was applied per gel lane. Apparent molecular weights were calculated from a graph of relative mobilities versus log mo-

Table 1
General features of Cu-adequate vs Cu-deficient rats

Diet	Body weight (g)	Liver Cu (μg/g)	Liver Fe (μg/g)	Hemoglobin (g/L)	Hematocrit (%)
CuD	270.8 ± 21.7*	1.665 ± 0.28†	451.5 ± 104.7*	74.45 ± 9.6*	22.7 ± 2.81†
CuA	321.4 ± 29.2	13.83 ± 2.41	310.4 ± 80.18	200.8 ± 108.4	46.5 ± 4.13

Values are means ± SD, $n = 6$.
* Significantly different from the CuA group (*$P < 0.05$; †$P < 0.001$).

lecular weight for standard proteins. Slab gels (10 × 14 cm) were routinely stained in 0.25% Coomassie Brilliant Blue R-250 in 45% methanol / 9% acetic acid for 1 hour and de-stained overnight in the above solution without dye.

2.5. Immunodetection-dot blotting

Different quantities of HSB protein extracts were bound to a Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was incubated with 10% Carnation nonfat dry milk in TBS containing 0.1% Tween for 1.5 hour at room temperature. Affinity purified anti-collagen type I antibody from Biotrend (Destin, FL) and horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG were diluted in 0.1 TTBS (1:6000 and 1:5000, respectively). Detection of signal was performed by using an ECL plus kit (Amersham Pharmacia Biotech, Piscataway, NJ) with the Molecular Dynamics Image-Quant system (Sunnyvale, CA).

2.6. One-dimensional reverse-phase chromatography with on-line mass spectrometry

Coomassie-stained gel slices containing only the 132 kDa pink band from the CuD samples, along with slices

from the corresponding area of CuA gels, were sent to the University of Victoria (Victoria, BC, Canada) for determination of the protein species in the respective complexes by use of their Applied Biosystems/MDS Sciex Qstar hybrid LC/MS/MS quadrupole time-of-flight (TOF) system (service contract).

2.7. Electrophoretic gel mobility shift assay

Gel shift experiments were performed as previously described [22]. Briefly, HSB protein extract (4 μg) was incubated with 25,000 cpm of ^{32}P-end-labeled single-stranded DNA probe (5 fmol) in a total volume of 20 μL for 20 minutes on ice and then 15 minutes at room temperature. After incubation, the reaction mixture was separated by electrophoresis through a 5% nondenaturing polyacrylamide gel at 4°C. The image signals representing the DNA-

Fig. 2. Effect of copper (Cu) deficiency on type I collagen content of heart high-salt buffer (HSB) protein extracts. (*Panel A:*) Representative dot blotting analysis with two rats for each group. Experiments were repeated two times more with four additional rats for each group. (*Panel B:*) the content of type I collagen was quantified by the Molecular Dynamics Image-Quant system (Sunnyvale, CA). Values are means ± SD, $n = 6$. *Different from CuA group, $P < 0.00001$.
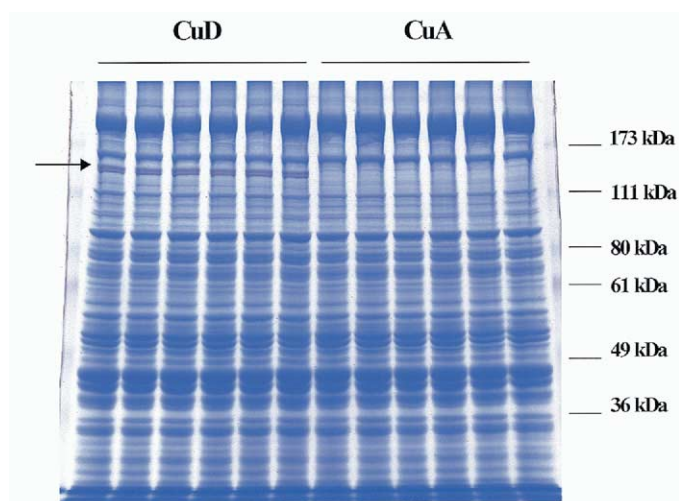
Fig. 1. Effect of copper (Cu) deficiency on protein species (50 μg protein each lane) analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. Heart high-salt buffer (HSB) protein extracts (six rats per group) were isolated from rats fed either a Cu-deficient diet (CuD) or Cu-adequate diet (CuA) for 5 weeks. The 132-kDa pink protein band is indicated by the arrow.
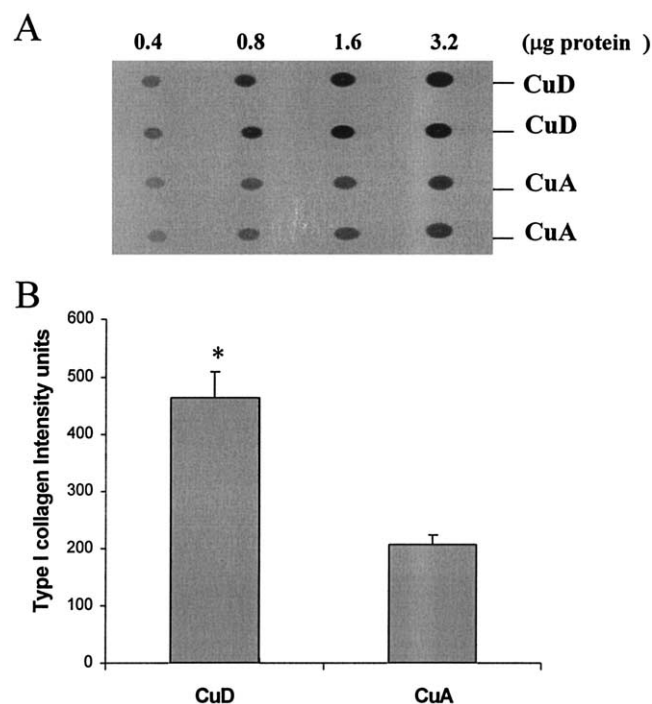
Table 2
Protein species at 132 kDa position of SDS-PAGE in CuA & CuD protein extracts

CuA protein extract accession_id	Name	Peptide	best_confidence_
gil12643614	Sarcoplasmic	AOGVVVATGVNTE	98
gil12643614	Sarcoplasmic	ISLPVILMDETLK	76
gil12643614	Sarcoplasmic	MNVFDTELK	92
gil12643614	Sarcoplasmic	NMLFSGTNIAAGK	86
gil12643614	Sarcoplasmic	VDQSILTGESVSV	94
gil12643614	Sarcoplasmic	VGEATETALTBLV	94
gil1091195	C protein [Mus musculus]	EGQPLAGEEVSIR	92
gil1091195	C protein [Mus musculus]	LTVELADPDAEVK	97
gil1091195	C protein [Mus musculus]	NPVGEDQVNLTVK	98
gil1091195	C protein [Mus musculus]	NSPTDTILFIR	97
gil1091195	C protein [Mus musculus]	VEFEBEVSEEGAQ	98
gil1091195	C protein [Mus musculus]	VIDVPDAPAAPK	97
gil20863388	(XM_134169) similar to adenine nucleotide translocase 1	DFLAGGIAAAVSK	71
gil20863388	(XM_134169) similar to adenine nucleotide translocase 1	EQGFLSFWR	76
gil20863388	(XM_134169) similar to adenine nucleotide translocase 1	YFPTQALNFAFK	98

CuD protein extract accession_id	Name	Peptide	best_confidence_
gil12643614	Sarcoplasmic	AOGVVVATGVNTEIGK	95
gil12643614	Sarcoplasmic	IGIFGQDEDVTSK	98
gil12643614	Sarcoplasmic	ISLPVILODETLK	71
gil12643614	Sarcoplasmic	MNVFDTELK	85
gil12643614	Sarcoplasmic	NMLFSGTNIAAGK	92
gil12643614	Sarcoplasmic	SEIGIAMGSGTAVAK	86
gil12643614	Sarcoplasmic	VDQSILTGESVSVIK	94
gil12643614	Sarcoplasmic	VGEATETALTBLVEK	95
gil1091195	C protein [Mus musculus]	LTVELADPDAEVK	98
gil1091195	C protein [Mus musculus]	NSPTDTILFIR	85
gil1091195	C protein [Mus musculus]	TSGGQSLAELIVQEK	95
gil1091195	C protein [Mus musculus]	VEFEBEVSEEGAQVK	98
gil20863388	(XM_134169) similar to adenine nucleotide translocase 1	DFLAGGIAAAVSK	92
gil20863388	(XM_134169) similar to adenine nucleotide translocase 1	EQGFLSFWR	71
gil20863388	(XM_134169) similar to adenine nucleotide translocase 1	YFPTQALNFAFK	92
gil2138932	(NM_028233) leucine rich protein LRP130	AGYPQYVSEILEK	97
gil2138932	(NM_028233) leucine rich protein LRP130	NVQGIIDILK	98
gil2138932	(NM_028233) leucine rich protein LRP130	TLLELIPELR	97
gil2506305	Collagen alpha 1 (I) chain precursor	GFSGLQGPPGSPGSPGEQ-GPSGASGPAGPR	76
gil2506305	Collagen alpha 1 (I) chain precursor	SAGVSVPGPMGPSGPR	92

protein complex were quantified and analyzed with a Molecular Dynamics Image-Quant system (Sunnyvale, CA). Sequence of the oligonucleotide probe used in the gel shift assays was 5'-dGAT(CTGCC)$_8$-3'.

2.8. Statistical analysis

Results are given as means ± SD. The Student *t* test for unequal variances was used to assess diet effects on hepatic Cu and Fe concentrations, hematocrit, hemoglobin, type I collagen content, and cytosine-rich DNA binding. Differences with a *P* value < 0.05 were considered to be significant.

3. Results

As shown in Table 1, body weight, hematocrit, hemoglobin, and liver Cu were lower, and liver Fe was higher, in CuD than in CuA rats. These data are characteristic of Cu-deficient rats, and our prior studies have shown that the Cu deficiency represented by such changes causes a significant depression of Cu concentration in the heart as well [23,24]. SDS-PAGE analysis showed a pink protein band at 132 kDa position in Cu-deficient but not in Cu-adequate HSB protein extract (Fig. 1). Immuno-dot blotting assay demonstrated that total type I collagen was increased by 110% in Cu-deficient relative to Cu-adequate HSB protein extract (Fig. 2). Furthermore, liquid chromatography–mass
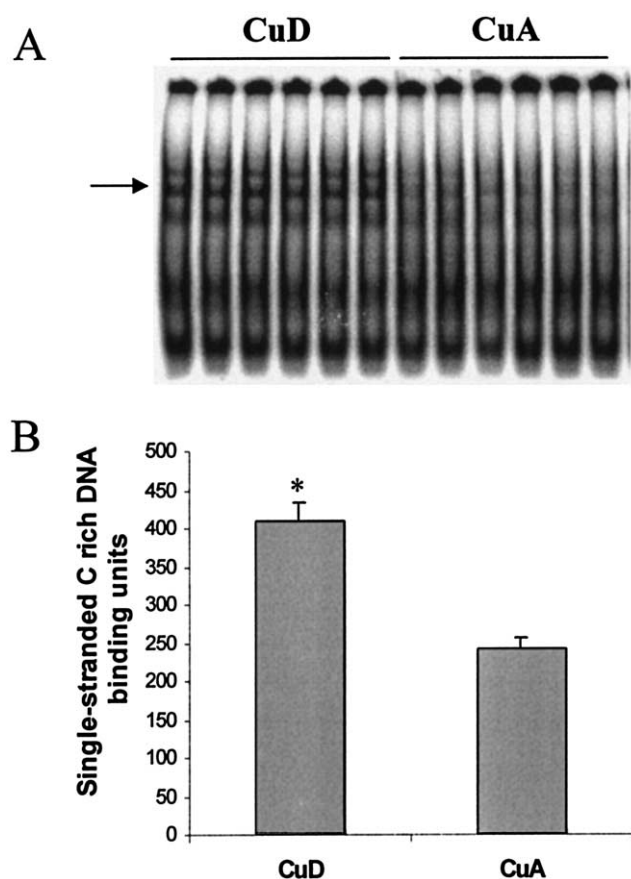
Fig. 3. Effect of copper (Cu) deficiency on single-stranded, cytosine-rich DNA binding. (*Panel A:*) electrophoretic mobility shift analysis of heart high-salt buffer (HSB) protein extracts from rats fed either a Cu-deficient diet (CuD) or a Cu-adequate diet (CuA) for 5 weeks (six rats per group). In each binding assay, heart HSB protein extract (4 μg) bound to the ^{32}P DNA probe (5 fmol). The specific binding position of the ^{32}P DNA and protein complex is indicated by the arrow. (*Panel B:*) Image signals representing the protein complex were quantified and analyzed with Molecular Dynamics Image-Quant system (Sunnyvale, CA). Values are means $\pm$ SD, $n = 6$. *Different from CuA group, $P < 0.00001$.

spectrometry analysis (Table 2) showed multiple protein species in the region of the 132 kDa pink protein band. Collagen α–type I precursor and LRP 130 were two additional species found in Cu-deficient HSB protein extract that were not present in Cu-adequate extract. Finally, gel shift analysis demonstrated that HSB protein extract from Cu-deficient rats bound to a single-stranded cytosine-rich DNA with higher affinity than did that from Cu-adequate rats (Fig. 3).

4. Discussion

Recently, large-scale expression analyses have indicated that not only fetal genes but also genes involved in signaling pathways and energy metabolism have been altered in hypertrophied hearts [25–27]. Because HSB protein extract contained enriched nuclear proteins and other cellular pro-

teins from other organelles such as mitochondria, protein profiles of HSB protein extracts from Cu-deficient/adequate hearts were examined in the present study.

First, with SDS-PAGE analysis, we detected a unique 132 kDa pink protein band in Cu-deficient HSB protein extract but not in Cu-adequate HSB protein extract. This observation suggested the presence of proline-rich proteins because only some proteins from brain (rubrophilin), collagens, histones, and parotid gland proteins are distinctly red when stained with Coomassie Blue [21]. It is extremely difficult to generate antibodies with specificities to collagens because collagens are highly conserved throughout evolution and are characterized by a "Glycine-X-Y" triple helical structure, whereas type-specific antibodies are dependent on nondenatured three-dimensional epitopes. Therefore, we used dot blotting analysis to confirm that the content of type I collagen was higher in Cu-deficient HSB protein extracts. This observation may be related to the finding of the reduced cardiac lysyl oxidase activity and altered collagen cross-links in Cu deficiency [28]. The impaired cross-linking may have allowed more collagen to be extracted during the high-salt buffer isolation procedure [28]. In agreement with increased collagen, analysis by liquid chromatography with mass spectrometry indicated that collagen α (I) chain precursor was one of the two additional proteins in Cu-deficient HSB protein extract. The data suggest that Cu deficiency, either directly or by its effect on lysyl oxidase activity, may up-regulate the transcription of pro-collagen α (I) chain gene, and/or may impair the conversion of collagen α (I) chain precursor to mature collagen α (I) chain.

The other important observation of this study is that LRP130 protein was identified as a major unique protein species in Cu-deficient HSB protein extract. Although we have not been able to obtain a good antibody to determine the exact increase in LRP130 protein in Cu-deficient HSB protein extract, the relative increase may be inferred from the increase in binding of Cu-deficient HSB protein extract to the single-stranded cytosine-rich DNA, as such binding is a known property of LRP130 [29–32]. LRP130, a protein containing nine pentatricopeptide repeat motifs, interacts with a single-stranded cytosine-rich sequence of hypervariable minisatellite in vertebrate genomes. Hypervariable minisatellite is frequently found in several types of tumors in humans and in experimental animals, and also in cultured cells treated with various carcinogens and ultraviolet irradiation [29–31]. In addition, LRP130 protein is an mRNA-binding protein likely involved with mtDNA transcript processing related to mitochondrial pathophysiology such as human cytochrome c oxidase deficiency [32]; the latter is consistent with the known role of dietary copper in cytochrome c expression and cytochrome c oxidase activity [1,8,33]. Taken together, our present findings not only demonstrate an additional feature of altered collagen metabolism with Cu deficiency, but also demonstrate for the first time that an increase in single-stranded cytosine-rich DNA

binding in Cu-deficient rat heart, which may have a potential to influence DNA stability and mRNA transcript processing.

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