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Research Paper

miR-122, a Mammalian Liver-Specific microRNA, is Processed from *hcr* mRNA and May Downregulate the High Affinity Cationic Amino Acid Transporter CAT-1

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NOTE

Supplementary information accompanies this manuscript. The supplementary Figures and text can be found at: http://www.landesbioscience.com/journals/rnabiology/changRNA1-2-sup.pdf

ABSTRACT

These studies show that miR-122, a 22-nucleotide microRNA, is derived from a liver-specific noncoding polyadenylated RNA transcribed from the gene *hcr*. The exact sequence of miR-122 as well as the adjacent secondary structure within the *hcr* mRNA are conserved from mammalian species back to fish. Levels of miR-122 in the mouse liver increase to half maximal values around day 17 of embryogenesis, and reach near maximal levels of 50,000 copies per average cell before birth. Lewis et al. (2003) predicted the cationic amino acid transporter (CAT-1 or SLC7A1) as a miR-122 target. CAT-1 protein and its mRNA are expressed in all mammalian tissues but with lower levels in adult liver. Furthermore, during mouse liver development CAT-1 mRNA decreases in an almost inverse correlation with miR-122. Eight potential miR-122 target sites were predicted within the human CAT-1 mRNA, with six in the 3'-untranslated region. Using a reporter construct it was found that just three of the predicted sites, linked in a 400-nucleotide sequence from human CAT-1, acted with synergy and were sufficient to strongly inhibit protein synthesis and reduce mRNA levels. In summary, these studies followed the accumulation during development of miR-122 from its mRNA precursor, *hcr*, through to identification of what may be a specific mRNA target, CAT-1.

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Reviews struggle to keep pace with the many exciting findings related to microRNAs (miRNA) that have been found in plants and animals.[1-9] These RNAs are of about 21–26 nucleotides in length and are derived by cleavage from much larger precursor RNAs that for the most part, seem to be noncoding polyadenylated RNA polymerase II transcripts.[10] The cleavage depends on the consecutive actions of at least two endonucleases. The first is Drosha, which acts in the nucleus and reduces the precursor to a fragment of about 60–70 nucleotides, which typically has the ability to fold into a hairpin structure in which the base-pairing in the stem is approaching 100%.[9, 11] This fragment is actively transported to the cytoplasm[12,13] where it is further cleaved by the enzyme Dicer, to release the miRNA species.[11,14] These miRNA can then act in two ways to reduce the expression of mRNA to which the miRNA contains some homology. The first way, seen more frequently in plants rather than animals, leads to the endonucleolytic cleavage of the target mRNA.[15] A second way, considered more common in animals, is that homology of the miRNA to target mRNAs is somehow able to interfere with the ability of that mRNA being translated.[8,9,16-18]

The expression of specific miRNA has in some cases proved to be temporal, leading to important roles in development.[1,19] In other cases, it has been correlated with expression in specific tissues,[20] implying roles in differentiation. Supportive of such roles is evidence that aberrations in the level of miRNA expression are implicated in some cancers.[21,22]

In mammals it has been estimated that there are at least 200 different miRNA species.[20,23-25] The studies described here refer to one specific miRNA, designated miR-122.[26] Uncovering of this species results from two independent studies.

One study dates from 2002, when Tuschl and colleagues reported an extensive cloning and sequencing project for the small RNAs present in various adult mouse tissues.[20] Many miRNA species were thus identified. miR-122 was found to be present only in liver tissue. Also it was the most frequent miRNA isolated from the liver. At that time, when the sequence of miR-122 was examined against the available nucleotide sequence database, the only match was a woodchuck sequence submitted in 1989.

At that time, Buendia and colleagues were studying genetic alterations in woodchuck liver tumors induced by a virus related to the human hepatitis B virus. In a majority of tumors, rearrangement and enhanced expression of N-*myc* or c-*myc* were found to result from viral insertional mutagenesis, but they characterized a chromosomal translocation in a unique tumor case, W64.[27] At the breakpoint, the 5'-end of a previously unknown gene,

which they named *hcr* (gi:51212), was recombined with the second exon of c-*myc*, correlating with a huge (over 50-fold) increase in c-*myc* expression.[28,29] The *hcr* transcript present in normal woodchuck liver was a 4.7 kb unspliced polyadenylated RNA. It is essentially noncoding, with no more than a very small (37aa) open reading frame located near the 5'-end, followed by an unusually large 3'-UTR.[29] The authors also were puzzled to find that the majority of this *hcr* RNA was located in the nucleus and had undergone endonucleolytic processing at several sites around 200 nt from the site of poly(A) addition. Within this 3'-fragment was the sequence that Tuschl and coworkers "discovered" as containing miR-122.[20]

Since then, we have reported studies of miR-122 as part of an examination of Dicer action on the RNAs of hepatitis delta virus. In that study, the detection of miR-122 in liver tissue and cultured liver cells was used as indirect evidence that Dicer was both present and active.[30] This was the first evidence for miR-122 expression in human cells.

The present study was initially undertaken to focus on several aspects of miR-122 expression. A sensitive and quantitative RNase protection assay was applied to determine the levels of miR-122 in different animal tissues and cell lines. The assays were also applied to reveal that miR-122 expression undergoes a specific turning-on in the liver of developing mouse embryos, and to quantify miR-122 in liver tumors, including the woodchuck tumor W64.

Whilst our study was underway, others reported theoretical attempts to predict the targets for mammalian miRNA species.[31-33] Uniquely, Lewis et al. predicted that the most likely mRNA target for miR-122 was a specific cationic acid transporter protein, CAT-1 (also known as solute carrier family 7 cationic amino acid transporter y^+ system member 1, or SLC7A1)(MGI:88117). CAT-1 also functions as the receptor for an endogenous ecotropic murine retrovirus associated with leukemia.[34] Their prediction made use of base pairing between miR-122 and the 3'-untranslated region of CAT-1. Also, they showed that the predicted pairing was conserved not only for human, mouse and rat, but also for fugu. In line with their prediction, it has been shown that expression of CAT-1 mRNA is unique in that it is detected in all adult mammalian tissues with the exception of the liver.[35, 36] As reported here, we set about to extend the predictions of Lewis et al. and also to obtain experimental evidence using quantitative assays for CAT-1 mRNA, *hcr*, and miR-122. We tested predicted miR-122 targets using reporter constructs. Our experimental results strongly support the interpretation that CAT-1 mRNA is an in vivo target for degradation directed by miR-122. In a more general view, our results show that a tissue-specific microRNA, acting post-transcriptionally, can contribute to the tissue-specific inhibition of a target RNA that is actively transcribed in almost all tissues.

MATERIALS AND METHODS

Cell and Tissues. Cell lines used were Huh7,[53] HepG2,[54] 293T,[55] AML-12,[56] and WC-3.[57] Primary human hepatocytes were provided by In Vitro Technologies. All mouse and woodchuck tissues were taken promptly post mortem, snap-frozen with liquid nitrogen, and later subjected to extraction of RNA.

Transfections. DNA constructs were delivered to cells using Lipofectamine 2000 (Invitrogen) according to the directions of the manufacturer. For the experiment shown in Figure 4E, a 22-nt 2'-O-methyl oligonucleotide exactly complementary to miR-122, as obtained commercially (Integrated DNA Technologies), was cotransfected (along with the specified DNA constructs) at a final concentration of 30 μM.

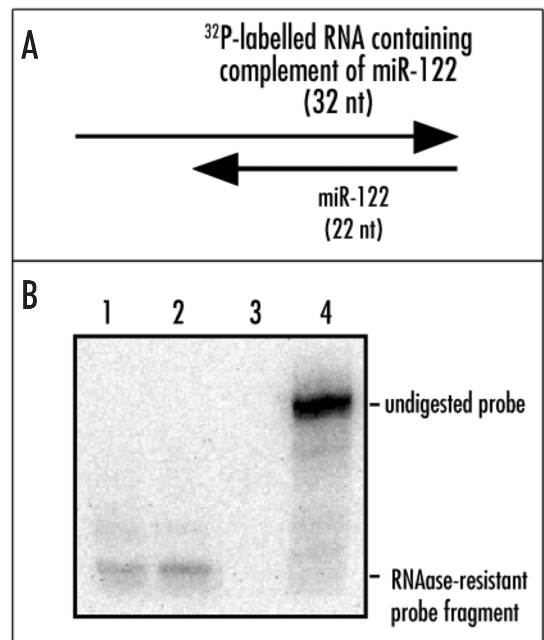

Figure 1. RNase protection assay for the detection of miR-122. As described in Methods, a 32 nt ^{32}P-labelled RNA probe was hybridized to either in vitro transcribed miR-122 RNA (lane 1), adult mouse liver RNA (lane 2) or without added RNA (lanes 3 and 4). The samples in lanes 1–3 were next digested with nucleases, and then all four samples were subjected to 15% polyacrylamide gel analysis in the presence of 8 M urea, followed by quantitation of radioactivity using a bio-imager (Fuji). Indicated are the positions of the undigested probe and the RNase resistant probe fragment.

RNA Extraction. Total RNA was extracted using Tri Reagent (Molecular Research Center) and quantitated using either a Nanodrop or the Agilent 2100 BioAnalyzer in combination with a RNA 6000 Nano LabChip.

Ribonuclease Protection Assay for miR-122. Our assay for detection of miR-122 was based on the *mir*Vana™ miRNA detection kit from Ambion. Typically, 0.1 or 1 μg of total RNA was hybridized to a ^{32}P-RNA probe, followed by RNase digestion, extraction, and finally polyacrylamide gel analysis and quantitation. Both the ^{32}P-labeled probe and the RNA standard for quantitation were transcribed using a *mir*Vana probe construction kit (Ambion).

Quantitative Real-Time PCR. Real-time PCR assays were performed using an ABI 7900 HT instrument. The primers and assay procedures are described in the Supplementary information.

Northern Analyses. Following glyoxalation and electrophoresis into a gel of 1.5% agarose, northern analysis was carried out using an RNA probes for mouse albumin and 28S rRNA.

Reporter Constructs for miR-122 Action. The strategy was as previously described.[58] Briefly, we inserted a putative target sequence into the 3'-untranslated region of a vector expressing the open reading frame for the 195 amino acid small protein of hepatitis delta virus. As a control, we used a vector that expressed the 214 amino acid large delta protein. These two plasmids were cotransfected and at day 3, the total protein was extracted and subjected to gel electrophoresis, to separate the two forms of delta protein, and then immunoblot, using a rabbit polyclonal antibody followed by ^{125}I-protein A (Amersham), to detect both proteins. Quantitation was via bio-imager (Fuji).

Exogenous Expression of miR-122. We inserted into the U6 vector pTZ U6+1,[40] between the U6 promoter and terminator for pol III, 160 nt corresponding to the sequences flanking the miR-122 from the woodchuck *hcr* gene. This strategy, following the report of Chen et al.,[43] is one which allows the in vivo transcript to be efficiently processed to release the miRNA species.

Table 1 **Quantitation of miR-122, *hcr* and CAT-1 in RNA Extracted from Tissues and Cultured Cells**

Source of RNA		miR-122 (copy number/ average cell)[a]	*hcr* (arbitrary units)[b]	CAT-1 (arbitrary units)[b]
Adult mouse tissues:				
	liver	66,000	740	40
	lung	< 50	0.69	300
	heart	< 50	0.02	830
	kidney	< 50	0.17	830
	spleen	< 50	0.37	2,100
	skeletal muscle	< 50	< 0.02	11
Human primary hepatocytes:		135,000	300	110
Established cell lines:				
Huh7	human well-differentiated hepatocellular carcinoma line	16,000	1,300	500
HepG2	human hepatoblastoma line	< 140	3.3	1,600
293T	human kidney line	< 140	< 3.0	930
AML-12	mouse hepatocyte line	< 140	8.0	47
WC-3	woodchuck chemically-transformed hepatocyte line	< 140	n.d.	n.d.

[a]miR-122 was quantitated by a RNase protection assay, as in Figure 1, allowing a deduction of the number of copies per average cell. [b]hcr and CAT-1 were assayed by real-time PCR as described in Supplementary Information, with the results expressed in arbitrary units. The woodchuck sample, indicated by n.d, was not done.

Prediction of miR-122 Targets. This was carried out as recently described.[38,39]

RESULTS

RNA Precursor to miR-122. The miRNA cloning study of Lagos-Quintana et al. reported that the mouse liver contains a total of three species related to miR-122.[20] miR-122 was the name given to the 22-nt species. miR-122a and miR-122b, respectively, were the names given to the related 23-nt species that contained an extra U or A at their 3'-end. At this time, the sequence databases for the genomes of human, chimp, mouse, rat, and woodchuck contain the miR-122a sequence but not that of miR-122b. Thus, the extra nucleotide of miR-122b might be a cloning artifact or possibly the consequence of a nontemplated nucleotide addition in vivo (Tuschl T, personal communication).

As presented in the Introduction, the likely RNA precursor for mammalian miR-122 is the essentially noncoding mRNA designated as *hcr*, as first found in woodchucks. Furthermore, as an extrapolation from the work of Lee et al., we might expect that this processing involves first action by Drosha, to produce a precursor of about 60–70 nucleotides, then action of Dicer, to release the miR-122.[11] Specifically for miR-122, others have predicted a stem-loop precursor of 66 nucleotides.[20]

With this in mind, we examined genomic DNA data for orthologous sequences in different animal species. In each case we considered a total of 160 nucleotides with the miR-122 sequence being central. We were able to align sequences for human, chimpanzee, cow, dog, mouse, rat, woodchuck, opossum, chicken, frog, Tetraodon and fugu. As shown in Figure 1S, we found that miR-122 (and miR-122a) is absolutely conserved in these 12 species. In the adjacent sequences comprising the predicted 66-nucleotide precursor[20] there was significant but less than 100% conservation. Outside of this region the conservation was real but much less.

When we applied S-fold to predict the structure of this 160-nucleotide region, we observed in all cases, the conservation of a stem-loop structure of at least 66 nucleotides, with the miR-122 sequence being embedded on one arm of this structure. We noted that within this 66-nucleotide region the sequence variations between the 12 species were almost exclusively in the nonpaired regions of this stem-loop structure (data not shown). Figure 2S shows the predicted structure for the woodchuck sequence. On this structure are indicated the three naturally-occurring *hcr* cleavage sites that were experimentally determined by Buendia and colleagues.[29] It can be seen that one of these sites corresponds exactly to the 3'-end of miR-122 and a second is 9 nucleotides 5' of the miR-122.

Experiments described subsequently further support the precursor role of *hcr* sequences in miR-122. Briefly, when we expressed in cells the abovementioned 160 nucleotides of woodchuck *hcr* sequence, we could obtain miR-122 species that were both abundant and functional.

Quantitation of miR-122. Previously we reported northern assays for miR-122 sequences in human liver cells.[30] However, for the present study we needed an assay that was more sensitive and quantifiable. We therefore chose an RNase protection assay. And, because of the above-mentioned sequence conservation, the assay did not need to be modified to study miR-122 in different species.

Figure 1 shows the detection of a protected fragment assayed on a denaturing polyacrylamide gel. Lanes 3 and 4, respectively, show the signal for the intact[32] P-probe with and without RNase digestion. Lane 2 shows the fragment protected by prior hybridization to 100 ng of total adult mouse liver RNA. Lane 1 shows a similar pattern for the protection achieved with 2 pg of an in vitro transcribed miR-122 RNA. Following such quantitation, if we assume that a liver cell contains 25 pg of RNA, we deduce at least 50,000 molecules of miR-122 per average cell. This calculation does not allow for variations in the amount of RNA per cell, or in this case, for the fact that only 70% of the cells in the adult liver are actually hepatocytes. For cell samples containing low levels of miR-122, the sensitivity of this assay could be increased by analysis of a larger (1 μg total) RNA sample, allowing the detection of as few as 50 copies of miR-122 per cell.

miR-122, *hcr* and CAT-1 Expression in Adult Mouse Tissues and Cultured Cells. With the RNase protection assay we quantitated miR-122 in adult mouse tissues. As summarized in Table 1, miR-122 was abundant in adult liver, with a range of 50,000–82,000 copies per average cell, but undetectable (< 50) in the other tissues tested. This tissue specificity confirms and extends earlier reports.[20,37]

Next we tested primary human hepatocytes and five different sources of cultured cells, as summarized in Table 1. As expected, primary human hepatocytes contained significant levels of miR-122. The amount was even more than for the adult mouse liver. Not surprisingly, the kidney cell line, 293T, contained no detectable miR-122. However, to our surprise only one of four hepatocyte cell lines tested, Huh7 cells, contained a detectable level of miR-122.

In addition to quantifying miR-122, we used real-time PCR assays for *hcr*, the precursor to miR-122, and CAT-1, the predicted target. Since the sequences of *hcr* and CAT-1, unlike miR-122, were different for human and mouse, we had to develop species-specific assays. In addition, from the studies of Moroy et al. we expected that most of the *hcr* transcripts detected in the liver would be already cleaved within their 3'-sequences, and yet, the

large 5'-fragment would still be much more abundant than the intact *hcr* RNA.[29] Therefore, we directed our real-time RT-PCR assay to sequences near the 5'-terminus.

Consider next the results in Table 1 for the *hcr* assays, especially in relationship to the corresponding data for miR-122. All these data are consistent with the expectation that *hcr* is the precursor to miR-122. Specifically, in the adult mouse tissues, *hcr* and miR-122 are only high in liver. In the primary human hepatocytes and the Huh7 liver cell line, both *hcr* and miR-122 levels were relatively high. In contrast for the two other liver cell lines (as for the kidney cell line) both *hcr* and miR-122 levels were much lower, in most cases below the detection limit.

Of the adult mouse tissues assayed for CAT-1, lung, heart, kidney and spleen all gave high levels. In contrast, levels in liver and skeletal muscle were significantly lower. The lower level of CAT-1 in liver is not inconsistent with regulation by high levels of miR-122. In contrast, for skeletal muscle, with undetectable levels of miR-122, the low level of CAT-1, could not be ascribed to negative regulation by miR-122. Similarly, not all the results for cultured cells gave a precise inverse correlation between miR-122 and CAT-1 levels. For example, with AML-12, a mouse liver cell line, both the miR-122 and CAT-1 levels were low. Thus, miR-122 could not have been negatively regulating the CAT-1. However, we do note that for primary human hepatocytes and the Huh7, a human liver cell line, the miR-122 levels were high while the CAT-1 levels were low. In addition, for HepG2, another human liver cell line and 293T, a human kidney cell line, the miR-122 levels were low while the CAT-1 levels were high.

miR-122, *hcr*, and CAT-1 Expression in the Liver during and Following Mouse Embryonic Development. Since studies with other miRNAs have detected changes during development we carried out similar studies using mouse liver samples taken both during embryonic development and for several weeks after birth. The liver bud begins to develop in the mouse embryo between embryonic days 8.5 and 9.5. Liver buds were collected at day 9.5 and found to be negative for miR-122 in our RNase protection assay (data not shown), but were positive in early fetal livers by day 12.5. For each of the days out from 12.5 to birth, and for days 1, 7 and 21 post natal, the amount of miR-122 increased with time of development, almost reaching a plateau level just before birth, and increased but at a much slower rate, after birth (Fig. 2A). Relative to the value at 21 days, the half-maximal value was reached at about 17 days post conception. In the livers from embryos taken early, at days 14.5 and 15.5, we attempted to crudely enrich for hepatocytes relative to blood forming cells. After the liver was minced, a brief sedimentation gave a pellet enriched for hepatocytes. In this pellet, the amount of miR-122 per mass of total RNA, was about 2-fold higher than for the supernatant (data not shown). This observation supports the interpretation that even at this early time of liver development, the miR-122 was being primarily detected in hepatocytes. In addition, it allows us to infer that miR-122 levels, per hepatocyte, are still much lower than the values observed later in development.

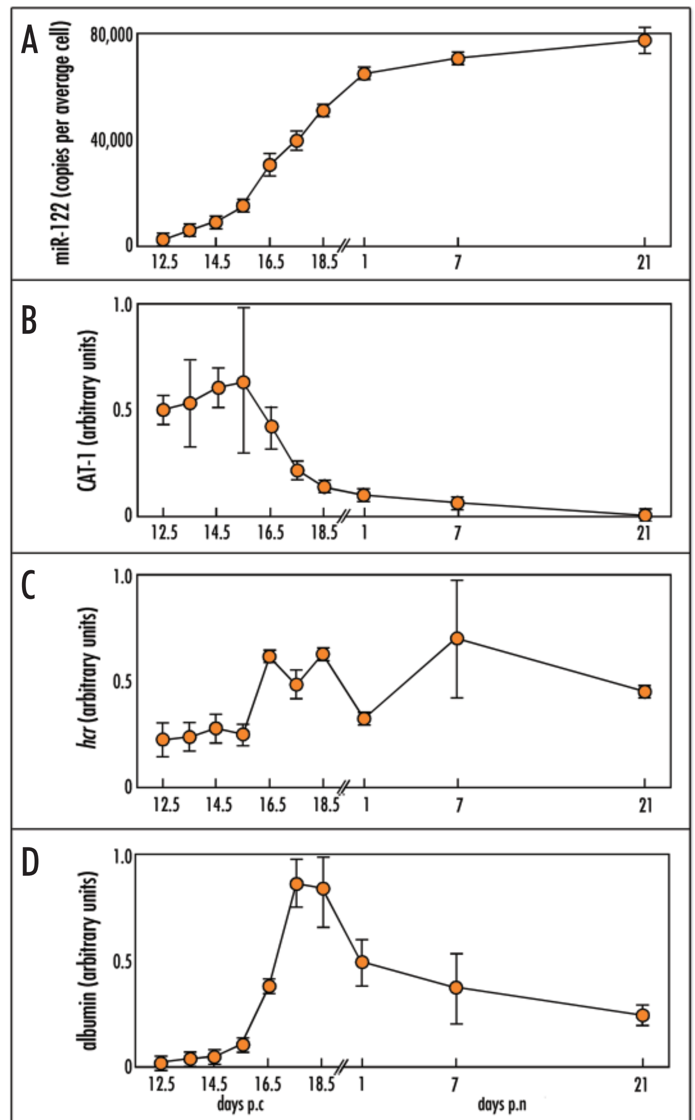

Figure 2. Assay of RNAs expressed in the liver during and following mouse embryo development. Liver tissue was taken post mortem, at the times indicated and total RNA extracted. (A) miR-122 was assayed as in Figure 1. (B) Mouse CAT-1 was assayed by real-time RT-PCR. (C) Mouse *hcr* was assayed by real-time PCR. (D) Mouse albumin was assayed by northern analysis. The filter was rehybridized to detect 28S rRNA and allow normalization. In (A) the levels are expressed as molecules per average cell while in (B–D) we use only arbitrary units. In each panel, each plotted point represents the average of values from two animals, with error bars expressing the range of the values.

Because miR-122 expression initiated after the formation of the liver bud (day 12.5), we hypothesized that it might play a role in modulating some of the diverse genes that are expressed during fetal liver development and then decreased with time of development, in a virtually reciprocal fashion to miR-122 expression. In addition to the prediction by Lewis et al. that miR-122 might target CAT-1, it was already known that during embryonic development, the levels of CAT-1 mRNA were specifically decreased in the liver. As shown in Figure 2B, we confirmed with quantitative RT-PCR assays that the mouse CAT-1 levels were in fact highest in embryonic liver. Furthermore, while the level at birth was down by about 70%, the level continued to decrease, and was down 97% by day 21 after birth.

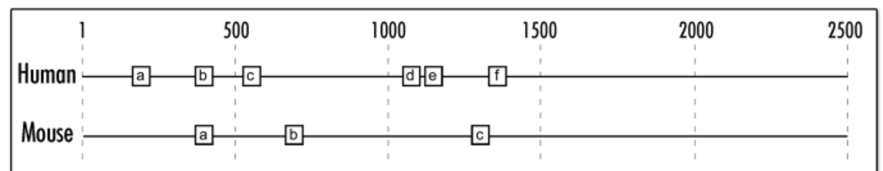

Figure 3. Predicted target sites for miR-122 in the 3'-untranslated regions of human and mouse CAT-1 mRNAs. Detailed information on the location of these sites is presented in Figure 3S.

The correspondence between the time-dependent increase of miR-122 and the decrease of CAT-1 was supportive of the prediction that CAT-1 was a direct target of miR-122. If so, the data are consistent with the interpretation that the inhibition was predominantly at the level of mRNA degradation. (Direct evidence for this is presented later in Fig. 4D).

In addition to miR-122 and CAT-1 we assayed *hcr* and albumin mRNAs. As shown in Figure 2C, we found *hcr* was expressed even at early embryonic times and then increased only several-fold during development and post-natal life. This range of levels was less than that observed for

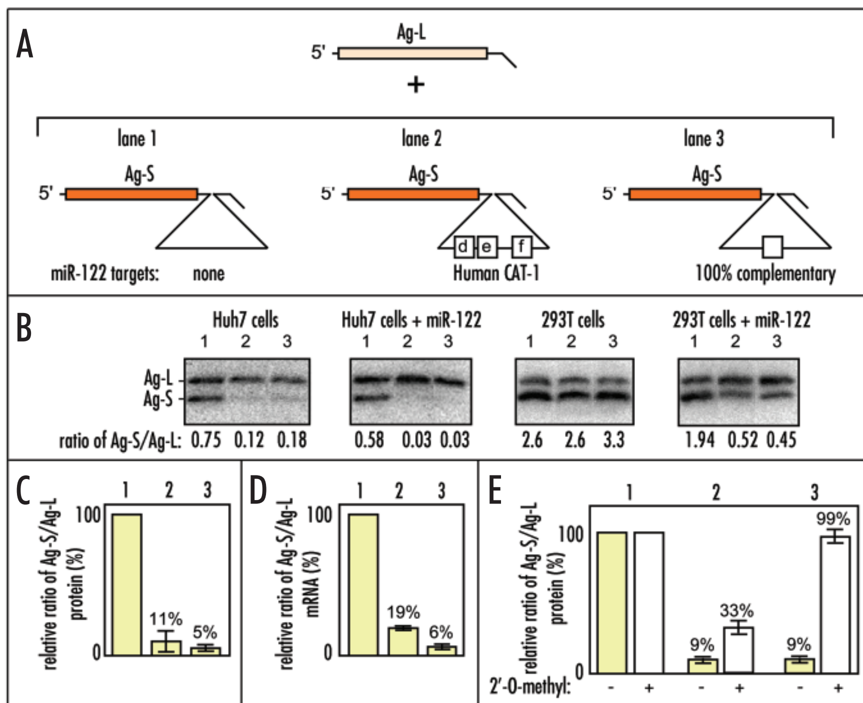

Figure 4. Action of miR-122 on reporter constructs containing predicted target sequences. (A) The open reading frames for the small and large forms of the delta protein (δAg-S and δAg-L, respectively) as were inserted into expression vector pcDNA3. Then, into the 3′-untranslated region of the vector for δAg-S, were inserted potential miR-122 target sequences. Lane 1 is a negative control, with no target. Lane 2 is a 400-nucleotide region of human CAT-1 with three potential miR-122 target sites, indicated in d–f (Figs. 3 and 5). For lane 3, the insert contains a single target with 100% complementarity to miR-122 (Fig. 5). (B) Examples of the immunoblot reporter assay. As indicated, Huh7 or 293T cells were cotransfected with a pair of such constructs along, without or with the U6 construct to enhance the accumulation of miR-122 sequences. At day 3 after transfection the total protein was examined by immunoblot to detect δAg-S and δAg-L. (C) The evaluation of seven such immunoblot assays using Huh7 cells that were also expressing exogenously provided miR-122. The vertical axis represents the ratio of δAg-S to δAg-L protein detected by immunoblot, normalized to the value for lane 1, as 100%. The error bars represent the range from seven assays. (D) The results of quantitative real time PCR to determine the ratio of the specific target mRNAs. The error bars represent the range from two assays. (E) Shows the ability of a 2′-O-methyl oligonucleotide complementary to miR-122, to interfere with the action in Huh7 cells of endogenous miR-122 against each of the three reporter constructs, as assayed by immunoblot. The vertical axis represents the ratio of δAg-S to δAg-L protein detected by immunoblot, normalized to the values for lane 1, as 100%.

miR-122. As a control these same RNA samples were assayed for expression of albumin; we observed a significant increase during embryonic development with a modest decrease beginning around the time of birth (Fig. 2D).

Prediction of miR-122 Target Sites on CAT-1 RNA. Applying algorithms previously used to predict miRNA targets in *Drosophila*[38] and human,[39] we examined the mRNA sequences for human and mouse CAT-1 for potential miR-122 target sites. These mRNAs each have a 3'-untranslated sequence of about 2 kb. Multiple sites were located, with results expressed fully in Figure 3S, along with an indication of the nucleotide sequence conservation between human and mouse. The six sites predicted for human CAT-1, are indicated as a–f in Figure 3. (Two additional sites were found, one in the 5'-untranslated region and another in the open reading frame.) Sites d and f were as predicted by Lewis et al.[31] For the mouse CAT-1 only three sites, indicated as a–c, were found. The site a, corresponded to one of the sites predicted for human CAT-1, and was the only mouse site predicted by Lewis et al.

Evidence that Endogenous and Exogenous miR-122 Can Act on Reporter Constructs. To more directly assess whether CAT-1 is an miR-122 target, we made use of chimeric mRNA reporter constructs. This strategy is represented with important examples in Figure 4. We assembled two expression vectors containing sequences of hepatitis delta virus. These express mRNAs that encode either the 214 amino acid, large delta protein (δAg-L) or the 195 amino acid, small delta protein (δAg-S). These two proteins have the same N-terminal sequence and can be quantitated in an immunoblot using a polyclonal rabbit antibody. As shown in Figure 4A, we added to the 3'-untranslated region of the δAg-S construct an acceptor sequence for potential targets for miR-122. Lane 1 is a negative control with no added target. Lane 2 is a 400 nt patch of human CAT-1. It contains three predicted target sequences (described in Figs. 3 and 5 as d–f). Lane 3 is a positive control, with a single target that is 100% complementary to the sequence of miR-122 (see Fig. 5).

Three days after cell transfection with a pair of such expression plasmids, we used an immunoblot to determine the ratio of δAg-S to δAg-L as a measure of the inhibition attributable to the presence in the δAg-S construct of the putative miR-122 target sequence. An advantage of this cotransfection strategy is that, independent of transfection efficiency or gel loading, we can measure the inhibition by quantitating δAg-S accumulation relative to that of δAg-L, as an internal control. It should be noted that in this reporter assay we can detect inhibition by miRNA whether it occurs by direct destabilization of the mRNA target or by an inhibition of translation.

The reporter assays were performed under four different conditions of miR-122 expression. From Table 1 it was known that Huh7 contain a significant endogenous level of miR-122, while 293T cells have none. Data are shown in Figure 4B for transfections into Huh7 and 293T cells, without and with the provision of exogenous expression of supplementary miR-122. In order to increase the level of miR-122 we used cotransfection with an expression vector containing 160 bp of the woodchuck genomic *hcr* sequence surrounding miR-122. These sequences were inserted into a vector which uses pol III transcription mediated via a U6 promoter and terminator sequences.[40] We were thus able to confirm using our RNase protection assay and also by northerns, that the level of miR-122 could be increased 3-fold in Huh7 cells, from about 16,000 to about 50,000 copies per average cell. The change was much more dramatic for 293T cells, raising the level from < 140 to about 50,000 copies per average cell (data not shown). Incidentally, these results provide the first experimental evidence that *hcr* sequences are processed in vivo to release miR-122.

For each of the four panels of Fig. 4B, lane 1 shows the results of the reporter assay for the negative control, of a δAg-S construct with no miR-122 target. Lane 2 shows data when the added target has the three predicted targeting sites in the human CAT-1 gene (as indicated in Fig. 3). In these studies, 293T cells without added miR-122, acted as a negative control for miR-122 action. Relative to these 293T cells, it can be seen that the Huh7 cells strongly inhibited the expression of the reporter with the human CAT-1 targets. Moreover, when miR-122 levels in Huh7 cells were increased with the U6 plasmid, there was an increased inhibition. Also the 293T cells, when provided with an additional source of miR-122, gave significant inhibition.

Finally, lane 3 shows the results for the perfect target, that is, one with 100% pairing to miR-122 (as indicated in Fig. 5). As expected, this like lane 2, showed no inhibition for 293T cells, but inhibition in Huh7 cells, and also increased inhibition for both Huh7 and 293T cells when extra miR-122 was provided.

The reporter construct tested in lane 2 of Figure 4 contains 400 nt of human CAT-1 sequences within which are three predicted miR-122 target sites. The data in B show the amount of the translated reporter protein from

this construct was reduced in both Huh7 cells, that have an endogenous level of miR-122, and in the 293T cells that have been transfected to express an exogenous level of miR-122. C shows the average results of seven experiments in which this construct was tested in Huh7 cells for the combined action of endogenous and exogenous miR-122. The inhibition was 89% with a range of ± 8%.

miRNA are considered to be able to act directly at either the translational level or directly at the level of target RNA cleavage.[9] Therefore, in order to determine which mechanism(s) applied in our studies we made use of quantitative real-time PCR assays. The results, as summarized in D, show that 81% inhibition was at the mRNA level. Thus, from a comparison of lane 2 in C and D we would conclude that the majority of inhibition seen in the protein assay was indirect, and due to loss of the mRNA.

In lane 3 of C and D, for a single target sequence with 100% complementarity to miR-122, there was relatively more inhibition, 94%. Like lane 2, this also showed inhibition consistent with mRNA loss. Such a conclusion is totally as expected from the findings of others with siRNA, and with most miRNA species in plants, that 100% complementarity will lead to targeted RNA cleavage and degradation.

From these studies we can deduce that miR-122, either endogenous or exogenous, are able to target the reporter construct containing CAT-1 sequences. As a further test of this interpretation we made use of a 2'-O-methyl oligonucleotide exactly complementary to miR-122. Others have shown that such oligonucleotides can reverse the action of siRNA and miRNA.[41,42] Huh7 cells were cotransfected with the reporter constructs, with or without the 2'-O-methyl oligonucleotide, and after 3 days an immunoblot was used to detect the reporter proteins. As shown in Figure 4E, the modified oligonucleotide was able to give significant reversal of action on CAT-1 sequences (lane 2). For the perfect target the reversal was virtually 100% (lane 3), that is, to the level achieved for a reporter with no miR-122 target (lane 1). These data further support the interpretation that much of the action against the CAT-1 construct as expressed in Huh7 cells is directly mediated via the endogenous miR-122. However, because the recovery was not 100% for the CAT-1 target in these Huh7 cells, we cannot exclude the possibility of contributions from additional activities, such as microRNAs other than miR-122. This caveat does not apply to the earlier results for miR-122 exogenously expressed in 293T cells; in that situation all the effects could be ascribed to the exogenous miR-122.

The targeting data for Figure 4, lane 2, apply to a sequence of 400 nt that contains three putative sites for miR-122. Therefore, we next tested these three sites individually using the same reporter strategy. The results are presented in Figure 5 along with the testing of a fourth site, one that is shared both by the human and mouse CAT-1 mRNA (Fig. 3). We thus found that when so tested, the four sites gave no or at most, only modest inhibition. Shown in Figure 5 are the predicted negative free energies for each of these targets in interacting with miR-122. Although this is a small data set, there is some correlation between activity and calculated stability. Certainly for the control target with 100% complementarity to miR-122 there is both highest negative free energy and highest level of inhibition.

While predicted negative free energy might be an indicator of target activity, the earlier results in which the three human CAT-1 targets were linked, there was a significant synergy. This is consistent with previous reports where multiple copies of a single target were found to produce synergy of miRNA action.[18]

We note that in our studies, to achieve inhibition of the target sequences, the stoichiometry of target to miR-122 effector was also important. As shown in Figure 4B, greater effects were seen when we increased the level of miR-122. Also, even for these data we first had to reduce the level of expression of the constructs containing the target sequences (data not shown). Therefore, we would anticipate that if relatively greater excesses of miR-122 to target could be achieved, even greater levels of inhibition would be observed.

Decreases in miR-122 Expression in Liver Tumor Tissues. As described in the Introduction, miR-122 was first found in the sequence of the *hcr* RNA. In one particular woodchuck tumor, W64, this actively transcribed liver-specific gene was translocated to in front of the second exon of c-*myc*.

		-ΔE (kCal/mol)	inhibition to (%)
perfect target	5'-CAAACACCAUUGUCACACUCCA-3'		
miR-122	3'-GUUUGUGGUAACAGUGUGAGGU-5'	43.30	5
Human CAT-1-d	5'-ACAGUCCAUGAAAUUGUGACACUCCAC-3'		
miR-122a	3'-UGUUU-GUGG--UAACAGUGUGAGGU-5'	25.34	100
Human CAT-1-e	5'-GGAAAUGGCCA-UGUCAUCACCCCU-3'		
miR-122a	3'-UGUUUG-UGGUAACAGU-GUGAGGU-5'	16.91	100
Human CAT-1-f	5'-GCCAGCACCAUUUCACACACACUCCU-3'		
miR-122a	3'-UGUUUGUGGUAACA---GUGUGAGGU-5'	26.02	45
Mouse CAT-1-a	5'-GCAU-CGCCCAUGUGCACACUCCAG-3'		
miR-122a	3'-UGUUUGUGGUAACA-GUGUGAGGU-5'	28.70	56

Figure 5. Putative target sequences for miR-122 action. Shown first is the binding of miR-122 to its 100% complement, the perfect target. Beneath this are three targets for miR-122a within the 3'-untranslated region of human CAT-1 mRNA and one from mouse CAT-1. Shown in the second column, are the negative free energies for the interaction between the microRNA and the predicted targets.[39] The third column, lists the observed inhibitions when each of these targets was inserted into a reporter construct and tested in Huh7 cells along with exogenous expression of miR-122, as used in Figure 4.

We reexamined the RNAs extracted from the tumor and nontumor tissue of this animal and found that the level of miR-122 was reduced 2-fold (data not shown). This was consistent with the known chromosome translocation.

We next tested whether changes in the level of miR-122 might also occur in other woodchuck liver tumors. We tested another 10 pairs of tumor and nontumor tissue taken from woodchucks chronically infected with woodchuck hepatitis virus, as studied recently at the Fox Chase Cancer Center. However, in none of these was there as much a change as 2-fold in the miR-122 levels between the tumor and nontumor tissues (data not shown). We can only conclude that changes of 2-fold in miR-122 accumulation were not a common event (< 10%) in these woodchuck liver tumors. In addition, while others have already assembled clear cases of altered miRNA levels contributing to cancer in animals[22] we must admit that for the W64 liver tumor, we have no evidence for or against the hypothesis that this reduction in miR-122 level per se, contributed to any aspect of the development of the liver tumor.

DISCUSSION

Many miRNA have now been shown to be developmentally controlled in plants, worms, and flies. Recent studies have also shown miRNA species that are developmentally controlled in mammals. Two studies show regulation of microRNAs during brain development.[19,37] A recent study has shown that in mouse, miR-181 modulates hematopoietic lineage differentiation.[43] Our studies with miR-122 are in this respect similar. However, our results have added value in that we have been able to follow not only expression of *hcr*, the precursor to miR-122, but also to follow CAT-1, the putative target. CAT-1 is an essential gene; homozygous knockout mice are severely anemic, runted and die within 12 h of birth.[44,45] CAT-1 is universally expressed in mammalian tissues with the dramatic exception of the adult liver. And yet, in the fetal liver it is strongly expressed.[35]

Several lines of evidence can be assembled for the claim that CAT-1 is a target for miR-122. (i) Theoretical predictions by Lewis et al. and also as presented here, suggest that CAT-1 is the most likely expressed genomic target for miR-122. (ii) Adult liver is the major site for miR-122, and yet conversely, CAT-1 mRNA is reciprocally

expressed, that is, in virtually all adult tissues with the striking exception being the liver.[35] (iii) During mouse embryonic development, miR-122 becomes expressed in the liver, as CAT-1 mRNA begins to decrease (Fig. 2). (iv) For the primary human hepatocytes and the human cell lines, there was again a reciprocal expression of miR-122 and CAT-1 (Table 1). That is, the primary cells had the highest levels of miR-122 and the lowest level of CAT-1, and yet for three cell lines (Huh7, HepG2, and 293T) as the levels of miR-122 decreased relative to the primary cells, there was a corresponding increase in the levels of CAT-1 expression. Thus, like the in vivo developmental profiles, the cell lines also exhibit an inverse correlation between the presence of miR-122 and CAT-1 mRNA. (v) Finally, there are reconstruction data with reporter constructs. When those regions of the human and mouse CAT-1 genes that are predicted to be miR-122 targets were added into the 3'-untranslated region of a reporter gene, the expression of that reporter became sensitive to inhibition by the presence of enhanced levels of miR-122 (Figs. 4 and 5). In these experiments we were able to show by quantitative RT-PCR that the action of the miR-122 on the reporter was primarily at the level of mRNA degradation (Fig. 4D). This result is a critical component of the inverse correlations cited above in (iii) and (iv).

Further comment needs to be made regarding our finding that miR-122 is acting at the level of destabilizing the CAT-1 mRNA. We know that our predicted miRNA targets have less than 100% pairing with the miR-122 (Fig. 3S and Fig. 5). Most studies find that in the absence of 100% pairing, inhibition is directly at the level of translation.[9,18,46] Of course activity on CAT-1 mRNA might be a special case; especially when we find that there are multiple sites that seem to act synergistically (Fig. 4). Furthermore, we would point out a finding from what have been considered as off-target effects for siRNA. It is that even with substantial mis-pairing between the siRNA and its off-target mRNA, the accumulation of that mRNA is reduced.[47]

Our observation of the dramatic synergy between just three miR-122 targets on CAT-1 mRNA (Fig. 4), emboldens us to propose the hypothesis that miR-122 is both necessary and sufficient for negative regulation of CAT-1 in mammalian liver cells. However, an important caveat is that we have not demonstrated miR-122 action on the full-length endogenous CAT-1 mRNA. Furthermore, it might still be that in certain situations, miRNAs other than miR-122, might target CAT-1 and contribute to its regulation. We do note that in adult mouse liver the 97% inhibition of CAT-1 (Fig. 2) is much greater than we achieved with the reporter constructs (Fig. 4). This could be due to differences in stoichiometry for the two systems. Alternatively, it might be that in vivo there may be additional inhibitory effects. For example, one could speculate that at the chromosomal level there might be miR-122 directed effects at the CAT-1 gene. These could be directed methylation of histones and/or methylation of the DNA, contributing to a specific transcriptional silencing.[48] Finally, while our data strongly support the interpretation of CAT-1 as a target for negative regulation by a microRNA in mammalian liver, further studies will be needed to evaluate the biological relevance of this regulation.

Are there other mRNA targets for miR-122? Lewis et al. did predict two targets, each less likely than CAT-1, as judged by conservation between species, ranging from human to mouse, rat, and fugu. Smalheiser et al. also predicted several miR-122 targets but failed to predict CAT-1.[33] We have predicted several interesting targets other than CAT-1. One is the gene for N-*myc*, a gene frequently rearranged in liver tumors induced in the woodchuck by woodchuck hepatitis virus.[49, 50] The other, is a gene referred to as "downregulated in liver malignancy."[51]

It is striking to point out that *hcr*, the precursor to miR-122, which was discovered as a gene rearrangement in a woodchuck liver tumor,[29] is a unique gene present only on chromosome 18 for human, mouse and rat. In humans, *hcr* is located at a position now designated as 18q21.31. Furthermore, both the primary sequence and the predicted secondary structure of the *hcr* mRNA sequences flanking miR-122, are conserved in 12 different animal species, from mammals to birds and fish (Fig. 1S). The early studies showed that for the woodchuck, *hcr* is expressed specifically in the liver.[29] We have confirmed that this is also true for the mouse (Table 1). In addition, we measured its expression during mouse development (Fig. 2C). While the pattern of change was somewhat similar to that of the miR-122 (Fig. 2A), there certainly was not a 1:1 correlation of *hcr* and miR-122 accumulation. Such differences may well be due to variations in *hcr* transcription and stability, processing of *hcr* to miR-122, and the ultimate stabilization of miR-122. Remember that in the early studies it was shown that in the adult woodchuck liver, the majority of the *hcr* transcripts were already processed at several discrete sites about 200 nt from the 3'-poly(A).[27] Intriguingly, one of these sites corresponds to the 3'-end of miR-122 (Fig. 2S). The present studies more extensively show that as little as 160 nt of sequence flanking from *hcr* when expressed in human liver cells is sufficient to allow the production of miR-122 that in turn, is able to lead to the destruction of a specific target RNA (Fig. 4). Further studies will be needed to determine what initiates the process by which the *hcr* transcripts are processed to release miR-122. Is it the availability of *hcr* transcripts or is it the initiation and/or completion of processing of existing transcripts?

In summary, the present studies have linked the developmentally controlled accumulation of miR-122 and its precursor *hcr*, to the ability to negatively regulate the stability of a specific target RNA, the mRNA for CAT-1. This microRNA provides a means to specifically down-regulate a target gene that is otherwise ubiquitously expressed. It may be a more expedient means of executing such regulation than having transcriptional control elements to promote high activity in all the cell types but not in just one or a few. In addition, since it has recently been shown in a diabetic rat model that CAT-1 mRNA is significantly up-regulated in the kidneys, thus contributing to the pathogenesis of hyperfiltration,[52] it might well be that delivery of miR-122 could be considered as part of a therapy.

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