Human RNase III Is a 160-kDa Protein Involved in Preribosomal RNA Processing*

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A human RNase III gene encodes a protein of 160 kDa with multiple domains, a proline-rich, a serine- and arginine-rich, and an RNase III domain. The expressed purified RNase III domain cleaves double-strand RNA and does not cleave single-strand RNA. The gene is ubiquitously expressed in human tissues and cell lines, and the protein is localized in the nucleus of the cell. The levels of transcription and translation of the protein do not change during different phases of the cell cycle. However, a significant fraction of the protein in the nucleus is translocated to the nucleolus during the S phase of the cell cycle. That this human RNase III is involved in processing of pre-rRNA, but might cleave at sites different from those described for yeast RNase III, is shown by antisense inhibition of RNase III expression. Inhibition of human RNase III expression causes cell death, suggesting an essential role for human RNase III in the cell. The antisense inhibition technique used in this study provides an effective method for functional analysis of newly identified human genes.

RNase III enzymes are highly conserved double-strand RNA (dsRNA) endoribonucleases expressed in most, perhaps all, living cells (1, 2). The COOH-terminal portion of the enzyme contains a dsRNA-binding domain, which has been found exclusively in proteins recognizing dsRNA. NMR methods have suggested that the dsRNA-binding domain has an α-β-β-α topology in which a three-stranded anti-parallel β-sheet packs on one side against the two α-helices (3). The enzyme also contains a catalytic domain independent from substrate-binding domain, which implies that substrate recognition is not necessarily coupled to catalysis. All RNase III species cloned to date contain a signature sequence (HNERLEFLGDS). In some species like Drosophila and Caenorhabditis elegans the enzyme contains 2 copies of the sequence (4) which may suggest that some RNase IIIIs may be capable of forming an active catalytic center as monomers. Deletion and mutation of this sequence abolishes the catalytic activity of the enzyme (5, 6). In Escherichia coli, RNase III forms a homodimer (7) and requires a divalent metal ion.

The enzyme produces 5′-phosphate, 3′-hydroxy termini and participates in RNA maturation and decay pathways by site specifically cleaving double-helical structures in cellular and viral RNAs. By comparing RNase III substrates, Zhang and Nicholson (8) showed several specific Watson-Crick base pairs at defined positions relative to the cleavage site. Introducing these disfavored base pairs into a model substrate inhibited efficient cleavage in vitro by interfering with RNase III binding. By comparing sequences of a large number of S. cerevisiae RNase III substrates and introducing mutations in the model substrate, Chanfreau et al. (9) suggested that yeast RNase III cleavage specificity may be based on recognizing consensus AGNN tetraloops and cleaving the substrate at a fixed distance from the tetraloop.

Multiple functions have been ascribed to RNase III. In E. coli RNase III has been reported to affect the expression of several phage, plasmid, and cellular genes (6, 10). The E. coli enzyme participates in the rRNA maturation process by processing 16 S and 23 S from the primary 30 S precursor (6). RNase III also plays a determinant role in control of the decay of messenger RNA and thus the level of corresponding proteins (2, 11). In yeast, it has been suggested that RNase III is a general factor in small nuclear RNA processing since enzyme cleavage sites were identified in several small nuclear RNA species (U1, U2, and U5). As these RNAs are essential components of the mRNA splicing apparatus (12–17), RNase III is thought to play a role in pre-mRNA processing. Yeast RNase III (RNT1) is also required for the synthesis of several small nuclear RNAs from large precursors (18–20). As most small nuclear RNAs are implicated in site-specific cleavage of precursor ribosomal RNA (20–22), the yeast enzyme is thought to play a key role in ribosome biogenesis (23, 24). Additionally, RNase III mutants of E. coli accumulate unprocessed pre-rRNA but are still viable because an alternative pathway for the processing of 16 S rRNA exists, and 23 S rRNA retaining unprocessed extensions at its 5′ and 3′ ends can assemble into functional ribosomes (25). On the other hand, the yeast RNase III (RNT1) is an essential gene in Saccharomyces cerevisiae.

To date, human RNase III has not been cloned. We now report the cloning and characterization of a cDNA that expresses a human RNase III. We demonstrate that the human enzyme is distinctly different from the homologues in other species and is involved in pre-rRNA processing.

MATERIALS AND METHODS

cDNA Cloning—An internet search of the XREF data base in the National Center of Biotechnology Information (NCBI) yielded a 393-base pair human expressed sequenced tag (GeneBank accession number AA033868) homologous to yeast RNase III (RNT1, GeneBank accession number U27016) and its C. elegans RNase III homolog (accession number Z81070). The first set of three oligonucleotide primers (NHII-2, NHII-4, and NHII-6) corresponding to the human expressed tag sequence was synthesized (Fig. 1A). By 3′ RACE (rapid amplification of 3′ cDNA), the human RNase III cDNA 3′ from the
expressed tag sequence was amplified by polymerase chain reaction, using human liver Marathon ready cDNA (CLONTECH, Palo Alto, CA) as templates, and NII-2/AP1 (for the first amplification) and NII-4/AP2 (for the second amplification) as primers (Fig. 1A). The standard polymerase chain reaction procedure was performed using native pfu DNA polymerase (Stratagene, San Diego, CA) and its reaction buffer. The annealing temperature range was 55–60 °C. The elongation time was approximately 6–8 min. The fragments were subjected to agarose gel electrophoresis in the TAE buffer, denatured in 0.5 M NaOH and then electronically transferred to a nitrocellulose membrane (Bio-Rad) for confirmation by Southern blot. Southern blots were hybridized using 3' P-labeled T7 transcript and unlabeled T3 transcript fragments as probes and subjected to DNA sequencing.

The confirmed fragments were excised from the agarose gel and purified by gel extraction (Qiagen, Germany), then subcloned into a zero-digested plasmid (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing.

A human liver cDNA plasmid Uni-ZAP library (Stratagene, La Jolla, CA) was screened using the RACE products as specific probes. The sequencing blunt vector (Invitrogen, Carlsbad, CA) and subjected to DNA sequencing. The sequencing of the positive clones were not long enough to encompass the full-length of human RNase III sequence, several sets of oligonucleotide primers based on the information from the cloned cDNA fragments were synthesized. Using these primers, several runs of 5' RACE were performed to clone three overlapping cDNAs, which resulted in full-length of cDNA (Fig. 1, A and B). The overlapping sequences were aligned and combined by the assembling program of MacDNASISv3.0 (Hitachi Software Engineering Co., America, Ltd.). Protein structure and analysis were performed by the program MacVector v6.0 (Oxford Molecular Group, United Kingdom). A homology search was performed on the NCBI database through the internet.

**Antibodies**—An SR domain peptide (H-CSRDSYDGRGTPRSRHRH-ESOSoutesica, 236–284) and an RNase III domain peptide (H-CRWEREQEPEPPDDITIYKIO, amino acids 1366–1394 (Fig. 1C)) were synthesized, purified (>90% pure), and conjugated to diphtheria toxoid with maleimidocaproyl-N-hydroxysuccinimide and used to raise polyclonal antibodies in rabbits. Anti-SR and anti-III peptide IgGs were affinity purified with SR and III peptides coupled to thiopeoply-Sephrose 6B, respectively (26).

**Western Blot**—Nuclear and non-nuclear fractions from HeLa cells were prepared as described (27). Whole cell, non-nuclear, and nuclear fractions were boiled in SDS sample buffer. Then the samples were separated by SDS-polyacrylamide gel electrophoresis using 4–20% Tris glycine gels (NOVEX, San Diego, CA) under reducing conditions. Molecular weight prestained markers were used (NOVEX) to determine the protein sizes. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane for immunoprecipitation using affinity purified anti-SR or anti-III peptide antibody at 5 μg/ml. The immunoreactive bands were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech) and analyzed using PhosphorImage Storm 860 (Molecular Dynamics, Sunnyvale, CA).

**Expression of GST-RNase III Domain Fusion Protein**—A cDNA fragment encoding the human RNase III-like domain (COOH-terminal 466 amino acids) was amplified by polymerase chain reaction and introduced into a BamHI site upstream and NotI site downstream. This fragment was further subcloned into the sites of the expression vector pGEX-4T-1 (Amersham Pharmacia Biotech) to produce the GST III fusion protein with glutathione S-transferase (GST) at its NH2 terminus. The identity of the construct was proven by DNA sequencing. The GST-RNase III fusion protein was expressed in E. coli strain BL21 and purified using glutathione-agarose (Amersham Pharmacia Biotech) under native conditions with B-PER bacterial protein extraction reagent (Pierce, Rockford, IL). Control GST protein was also prepared in parallel from the pGEX-4T-1 plasmid. The purified products were identified by Coomassie staining after 12% SDS-polyacrylamide gel electrophoresis and Western blot analyses with anti-RNase III peptide antibody (see above).

**In Vitro Cleavage of dsRNA**—The dsRNA substrate was generated by hybridization of two complementary strands of RNA produced with T7 and T3 polymerase transcription of the polylinker region of the pBluescript II KS(−) plasmid (Stratagene, San Diego, CA). The plasmid was digested with restriction endonuclease SstI or KpnI and further purified with phenol/chloroform extraction and ethanol precipitation. The SstI- or KpnI-digested plasmids were then transcribed using T7 or T3 RNA polymerase, respectively (Stratagene, San Diego, CA), with or without [α-32P]UTP. The resulting transcribed RNAAs (about 100 nucleotides) were purified by electrophoresis on 6% denaturing polyacrylamide gel. The 32P-labeled T7 transcript and unlabeled T3 transcript fragments were affinity purified with SR and III peptides coupled to thiopropyl-agarose/formaldehyde gel and transferred to Hybond-N (Amersham Pharmacia Biotech) followed by fixing using UV cross-linker (Stratagene, La Jolla, CA). The premade multiple tissue Northern blots were also purchased from CLONTECH (Palo Alto, CA). To detect RNase III mRNA, hybridization was performed by using 32P-labeled human RNase III cDNA probe (1.4 kb, clone 3-1) in Quik-Hyb buffer (Stratagene, La Jolla, CA) at 68 °C for 2 h. After hybridization, membranes were washed in a final stringency of 0.1 × SSC, 0.1% SDS at 60 °C for 30 min. To detect pre-RNA and rRNA, hybridization was performed by using 32P-end labeled oligo probes 5′ETS-1 (5′-CAGGACGACCTCT- CAGATCGTCTAGAGAGGTTTTCTTCA-3′), corresponded to 5′ETS and 5.8S-1 (5′-CATTAAATTTCTGACGCACGCTGTTCTTATCATCAG- ACGC-3′), corresponded to 5.8 S rRNA at 40 °C for 2 h and washed in 2 × SSC, 0.1% SDS at 40 °C for 1 h. Membranes were analyzed using PhosphorImage Storm 860 (Molecular Dynamics, Sunnyvale, CA).
RESULTS

Cloning of Human RNase III (Fig. 1, A and B)—The cloning strategy is described in detail under “Materials and Methods” and in the legend to Fig. 1. Using primers based on a search of the XREF database, an approximate 1.2-kb cDNA (clone U4) corresponding to the COOH-terminal portion of the protein was cloned by 3'- and 5'-RACE. Eight positive clones were isolated by screening a liver cDNA library with this clone. The two longest cDNA clones were used as templates for polymerase chain reaction (PCR) with 3'- and 5'-RACE or in detection on Southern blots (see “Materials and Methods”). The arrow indicates the region (not proportional) and direction. Primer AP1 and AP2 were provided with Marathon ready cDNA.

B. The full-length cDNA with 4764 base pairs was assembled with 5 major cDNA clones from either 5'-RACE or screening the library. Those are 3-1, 3-4, L40, 25, and 81, which covered positions are listed in the table. C. Schematic diagram of the human RNase III protein. P, proline-rich domain; SR, serine- and arginine-rich domain; RNase III, RNase III-like domain. Arrows identify the locations of SR and III peptides, which were used for antibody production. D. Primary amino acid sequence of human RNase III and comparison with C. elegans (Worm), S. pombe (PAC), and S. cerevisiae (RNT), and E. coli (RNC) RNase III. Bold letters, identical amino acids of human RNase III to other species. @@, putative catalytic center. HHH, a helix; BBB, a β sheet (dsRNA-binding region at COOH terminus). Amino acid identity of human RNase III to Worm (41%), PAC (17%), RNT (15%), and RNC (16%). * potential phosphorylation sites analyzed using OMIGA (Oxford Molecular Ltd.). E. Cleavage of dsRNA by GST-RNase III fusion protein. The duplex RNA (dsRNA) and single-stranded RNA (ssRNA) mixture was incubated with either GST-RNase III protein or GST alone as a control at 37 °C for 0, 4, 15, 60, and 240 min. The samples were then fractionated on a nondenaturing gel. Arrows indicate the position of ssRNA and dsRNA.

Fig. 1. Human RNase III protein. A, cloning strategy by 3'- and 5'-RACE. Sequence-specific primer sets listed in the table were designed based on the human expressed tag sequence or early cloned cDNA fragments. Those primers were all used in polymerase chain reaction for 3'- and 5'-RACE or in detection on Southern blots (see “Materials and Methods”). The arrow indicates the region (not proportional) and direction. Primer AP1 and AP2 were provided with Marathon ready cDNA. B, assembled diagram of full-length human RNase III cDNA clone. The full-length cDNA with 4764 base pairs was assembled with 5 major cDNA clones from either 5'-RACE or screening the library. Those are 3-1, 3-4, L40, 25, and 81, which covered positions are listed in the table. C, schematic diagram of the human RNase III protein. P, proline-rich domain; SR, serine- and arginine-rich domain; RNase III, RNase III-like domain. Arrows identify the locations of SR and III peptides, which were used for antibody production. D, primary amino acid sequence of human RNase III and comparison with C. elegans (Worm), S. pombe (PAC), and S. cerevisiae (RNT), and E. coli (RNC) RNase III. Bold letters, identical amino acids of human RNase III to other species. @@, putative catalytic center. HHH, a helix; BBB, a β sheet (dsRNA-binding region at COOH terminus). Amino acid identity of human RNase III to Worm (41%), PAC (17%), RNT (15%), and RNC (16%). * potential phosphorylation sites analyzed using OMIGA (Oxford Molecular Ltd.). E, cleavage of dsRNA by GST-RNase III fusion protein. The duplex RNA (dsRNA) and single-stranded RNA (ssRNA) mixture was incubated with either GST-RNase III protein or GST alone as a control at 37 °C for 0, 4, 15, 60, and 240 min. The samples were then fractionated on a nondenaturing gel. Arrows indicate the position of ssRNA and dsRNA.
clones, 3-1 and 3-4, correspond to the COOH-terminal region, 2636–3912 and 3350–4764 base pairs, respectively, of the full-length cDNA. With primers (3RACE1, 3RACE2, and 3RACE3) based on the NH2-terminal portion of the clone 3-4, 5' RACE was performed to clone a cDNA (clone L40) of approximately 1 kb, which encodes the middle part of the full-length cDNA. In the same way, a cDNA (clone 25) of the NH2-terminal portion was cloned. Using clone 25 to screen the liver library again, several clones were isolated, but none of these included additional NH2-terminal sequence. The most NH2-terminal clone is 328, which corresponds the sequence 799–2191 base pairs. So the last 5' RACE was performed with the primers (33G, 33H, and 33 Dec based on clone 25) and the NH2-terminal portion of the cDNA (clone 81) was generated.

These overlapping clones were sequenced and assembled to a full-length human RNase III cDNA with the total of 4764 nucleotides (Fig. 1B, GenBank accession number AF189011). The cDNA contained a coding sequence of 4125 (from 246 to 4370) nucleotides that encodes a 1374-amino acid protein. The calculated molecular mass of the protein is 160 kDa based on the prediction of the first methionine as the translation initiation site. The proposed initiation codon is in reasonable agreement with the mammalian translation initiation consensus sequence (Kozak sequence) (31). Northern hybridization analyses using clone 3-1 (Fig. 1B, 3' portion of cDNA) demonstrated that the human RNase III mRNA was approximately 5 kb. It was ubiquitously expressed in human tissues and cell lines (Fig. 2). When clone 25 (Fig. 1, 5 portion of cDNA) was used to probe Northers, equivalent results were obtained (data not shown). Compared with C. elegans, yeast, and bacterial RNase III, the full-length of human RNase III clone is substantially larger and contains multiple domains (Fig. 1C). The RNase III domain (approximately 426 amino acids) is located at the carboxyl terminus of the protein and is well conserved with other species such as C. elegans, yeast, and bacterial RNase III. This domain shares strong homology with C. elegans RNase III (41% amino acid identity, Fig. 1D). Both the human RNase III domain and C. elegans RNase III contain two RNase III signature sequences. The human RNase III domain also contains multiple potential phosphorylation sites. The full-length human protein also contains proline-rich (220 amino acids) and SR-rich (250 amino acids) domains near the amino terminus. The human RNase III domain also contains potential phosphorylation sites. The full-length human protein also contains proline-rich (220 amino acids) and SR-rich (250 amino acids) domains near the amino terminus (Fig. 1C). The SR and RNase III domains are separated by the 478-amino acid region.

The human RNase III domain cleaves dsRNA—To test whether the human RNase III domain can specifically cleave dsRNA, the RNase III domain-coding region was subcloned into a glutathione-S-transferase (GST) expression vector. The GST-RNase III fusion protein and GST alone were expressed, purified using glutathione-agarose, and analyzed by Coomassie Blue staining of the SDS-polyacrylamide gel electrophoresis and Western blot analysis with anti-human RNase III peptide antibody (data not shown). These studies showed that the human RNase III domain was greater than 85% pure. However, there was evidence of slight degradation during expression and purification. When incubated with labeled dsRNA and ssRNA, the GST-RNase III fusion protein preferentially digested the dsRNA without significant cleavage of ssRNA, while GST alone cleaved neither dsRNA nor single-stranded DNA substrate (Fig. 1E). Thus, the cleavage observed was not due to contamination with ssRNases or dsRNases from E. coli. Ribonucleases V1 (dsRNase), and T1 and A (ssRNases) were used as controls to confirm that the cleavage observed was dsRNA cleavage (data not shown).
As described under “Materials and Methods,” we incubated approximately 5–10 pmol of GST-RNase III domain with 5–10 fmol of dsRNA. Thus, the GST-human RNase III domain was perhaps 5–10-fold less active than the reported activity for yeast RNase III (23). However, the purification and reaction conditions and the substrate have not been optimized, and only the GST RNase III domain has been studied. Clearly, much more work is required with the full-length human enzyme before comparisons of the specific activities of the enzymes are possible.

**Human RNase III Is Localized in the Nucleus**—To determine the expression of the human RNase III protein, two anti-peptide antibodies were produced. The anti-III peptide antibody was derived from a peptide (amino acids 1356–1374) corresponding to the RNase III domain present in the COOH-terminal portion of the putative protein. The anti-SR peptide antibody was derived from a peptide (amino acids 266–284) corresponding to the SR-rich domain of the putative protein (Fig. 1C). Using these antibodies, Western blot analyses were performed to determine the size and localization of human RNase III. The anti-SR peptide antibody recognized a band in HeLa whole cell lysate with a size of approximately 160 kDa (Fig. 3A) which is near the calculated protein size confirming that the full coding region is expressed in HeLa cells. The anti-RNase III peptide antibody also recognized a 160-kDa protein in HeLa cells and that protein comigrated with the protein identified with the anti-SR domain antibody (data not shown). Similar experiments were performed using different human cell lines e.g. A549, T24, and HL60 with equivalent results (data not shown). To further confirm that the predicted protein is indeed expressed in human cells, the protein was partially purified with an RNase III domain peptide antibody column. The purified protein was then recognized on Western blots by both antibodies (data not shown). To determine the localization of the protein, nuclear and non-nuclear fractions from HeLa cells (Fig. 3A) and other human cell lines (data not shown) were prepared and equal amounts of proteins (40 μg) were analyzed by Western blots. RNase III was present primarily in the nuclear fractions (Fig. 3A). Non-nuclear fractions contained only trace amounts of protein, possibly due to the contamination during sample preparation. The anti-III peptide antibody gave results equivalent to those obtained with the anti-SR peptide antibody (data not shown). To better understand the localization of human RNase III, the protein was identified in cells by indirect immunofluorescence microscopy. The nuclei of HeLa cells were stained by both anti-SR (data not shown) and anti-III (Fig. 3B) antibodies, confirming that human RNase III is present in the nucleus. Fig. 3B also shows that RNase III is localized extensively in the nucleus and occasionally observed in nucleoli.

**Localization of Human RNase III to Nucleoli Is Cell Cycle Regulated**—Double thymidine treatment was used to synchronize HeLa cells to early-S phase (Fig. 4) (28). Two to four hours after releasing the thymidine block, HeLa cells entered S phase. Six to eight hours after releasing, HeLa cells entered G2/M phase. There were no significant changes in the mRNA or protein levels of the RNase III during pre-S, S, or G2/M phases (data not shown). However, the subcellular localization of the protein changed during the cell cycle. When the cells were treated with thymidine and synchronized in early S phase, RNase III protein was present only in the nucleus and not the nucleoli (Fig. 4B and F). After releasing from thymidine block, RNase III was translocated to nucleoli, reaching a peak at 4 h when cells were in S phase (Fig. 4C and G). At that time, RNase III was present both in the nucleoli and nucleus. The
protein was present in the nucleoli for approximately 8 h, and then disappeared from nucleoli as cells entered M phase (Fig. 4, D and H). Localization of RNase III in the nucleoli was confirmed by double staining with an anti-nucleolin monoclonal antibody (MBL, Watertown, MA) (data not shown). Moreover, there was no evidence of translocation of nucleolin to or from the nucleolus in these experiments.

Inhibition of Expression of RNase III with Antisense Oligonucleotides—To further evaluate the potential roles of human RNase III, antisense inhibition of expression of the protein was studied. To identify optimal sites in RNase III mRNA for antisense effects, 2'-methoxethyl chimeric antisense inhibitors to 10 sites in the mRNA were designed and screened for inhibition of RNase III (Table I). These antisense agents serve as substrates for RNase H when bound to RNA resulting in degradation of the RNA and have been shown to be highly specific when used under the conditions employed in our study (29). Table I shows that there was significant variation in the potency of the antisense inhibitors as a function of the site to which they were designed to bind in the RNase III mRNA. The most effective agent, ISIS 25691, targeted to a site in the coding region (nucleotide 3085–4004) of the mRNA, was selected for further study. Multiple control oligonucleotides were studied and the results from studies on ISIS 25691 (300 nM) compared with control. *12S RNA; ▲32S RNA; ■RNase III protein; ♦RNase III mRNA. Data shown are mean ± S.D. of triplicate determination. C, Western analysis of RNase III with anti-SR peptide antibody. D, indirect immunofluorescence staining of RNase III with anti-III peptide antibody after cells were treated with 100 nM oligonucleotides for 24 h.

Fig. 5. The effect of ISIS 25691 on the level of human RNase III mRNA (A and B), protein (B-D) and 12 S, 32 S pre-rRNA (B) in HeLa cells. Cells were treated with oligonucleotides for 24 h (A, C, and D) and 5–48 h (B). A, Northern hybridization of RNase III. *, accumulation of partially degraded RNase III mRNA. The level of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was used to normalize the amount of total RNA loaded. B, levels of RNase III mRNA, protein, and 12 S, 32 S pre-rRNA (see Fig. 6) in HeLa cells treated with ISIS 25691 (300 nM) compared with control. ●, 12S RNA; ▲, 32S RNA; ■, RNase III protein; ♦, RNase III mRNA. Data shown are mean ± S.D. of triplicate determination. C, Western analysis of RNase III with anti-SR peptide antibody. D, indirect immunofluorescence staining of RNase III with anti-III peptide antibody after cells were treated with 100 nM oligonucleotides for 24 h.

Increasing concentrations of ISIS 25691 caused increasing loss of RNase III mRNA, with 300 nM resulting in loss of more than 90% of the RNase III mRNA (Figs. 5A and 6C). The lower band on the blot that was hybridized by the probe may be a partially degraded mRNA fragment of RNase III (Fig. 5A). The control oligonucleotide, ISIS 27110 at 300 nM, had no effect on the RNase III mRNA level. ISIS 25691 at 300 nM suppressed RNase III mRNA levels in HeLa cells from 2 to 72 h after a single treatment (Fig. 5B, some data not shown). After treatment with ISIS 25691 at 100, 150, or 200 nM for 24 h, RNase III protein was reduced to 67, 44, or 19% of control, respectively (Fig. 5C). The level of RNase III protein was slightly reduced at 5 h after treatment and reached a maximum reduction at 18 h (Fig. 5B). Immunofluorescence staining showed that after
treatment with ISIS 25691, RNase III was dramatically reduced or absent in the nucleus and nucleoli (Fig. 5D).

After treatment of HeLa cells with ISIS 25691 at 300 nM for 18 h, the morphology of HeLa cells changed from fusiform to oval. After 24 h of treatment, approximately 5–10% of the cells detached from the plate and could be stained with trypan blue indicating cell death. The cells that remained attached to the solid substrate grew much more slowly than untreated cells. After 48 h, 40–50% of the cells treated with ISIS 25691 at 300 nM were dead.

These results suggest that RNase III is required for HeLa cell survival. The control oligonucleotide had no effect at any time or any concentration on cell morphology, RNase III mRNA or protein levels demonstrating the antisense effect was highly specific.

**Human RNase III Is Involved in Pre-rRNA Processing**—One function that has been attributed to RNase III in lower species is pre-rRNA processing (23, 32). As the human protein appeared to be translocated to and from the nucleolus during the cell cycle (Fig. 4), we evaluated its potential role(s) in human pre-rRNA processing. Two hybridization probes for human pre-rRNA were synthesized; 5′ETS-1, designed to bind to the 5′ external transcribed spacer (5′ETS, nucleotides 1393–1432) of human pre-rRNA (33) and 5.8S-1, designed to bind to 5.8 S rRNA (nucleotides 31–70) (34). Fig. 6 shows that when total cellular RNA (15 μg) from untreated HeLa cells was fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with [32P]-5′ETS-1, a band corresponding to 45 S pre-rRNA and a very faint band corresponding in mobility to 30 S (5′ETS-18S-ITS1) pre-rRNA were observed. When [32P]-5.8S-1 was used, bands corresponding to 45 S, 32 S (5.8S-ITS2–28S), and 12 S (5.8S-ITS2) pre-rRNA and 5.8 S rRNA were observed. At concentrations at which the antisense oligonucleotide, ISIS 25691 dramatically reduced the RNase III level, no effect on the 45 S pre-rRNA level was observed (Fig. 6, B and C). In contrast, the 5.8S-1 probe demonstrated that antisense inhibition of RNase III increased the levels of 32 S and 12 S pre-rRNAs (Fig. 6, B and C). The kinetic study shows...
that after treatment with ISIS 25691 at 300 nM, 32 S, and 12 S pre-rRNAs began to accumulate at 5 h and reached to peak at 18 h (Fig. 5B).

Fig. 7 provides further confirmation that RNase III is involved in pre-rRNA processing. In this figure, we compare the effects of 10 antisense oligonucleotides designed to bind to different sites in RNase III mRNA on RNase III mRNA levels to the effects of these oligonucleotides on accumulation of the two pre-rRNA species that accumulated after treatment with the most potent of the antisense inhibitors, ISIS 25691. As has been demonstrated repeatedly (35), different sites in RNAs are differentially sensitive to antisense inhibition, and, as expected, the potency of antisense inhibitors designed to bind to different sites in RNase III mRNA varied. The correlation between the reduction of RNase III RNA levels and the accumulation of both 32 S and 12 S pre-rRNAs was excellent (Fig. 7).

**DISCUSSION**

In this study, we cloned a cDNA encoding a human RNase III enzyme which is much more complex than either its prokaryotic or yeast homologue (5, 23, 36–40). If we consider the first translated methionine as the translation initiation site, the clone encodes a protein with a size of approximately 160 kDa. The presence of additional domains suggests that human RNase III may participate in protein-protein interactions that regulate its function and that it may be multifunctional. The SR and proline domains are usually present in proteins that play crucial roles in mRNA splicing (41). The fusion of SR and RNase III domains into a single protein suggests that human RNase III may be involved in a number of RNA metabolic events. The presence of multiple potential phosphorylation sites suggests that the enzyme may be regulated by phosphorylation.

The localization of the protein in both nucleus and nucleoli suggests possible involvement in both pre-mRNA and pre-rRNA processing. In E. coli, RNase III is associated with ribosomes in the cytoplasm (42). The studies reported here are the first to demonstrate that a eukaryotic RNase III is localized in the nucleus. In human cells, nucleoli undergo phases of condensation and dissociation as a function of the cell cycle. Nucleoli dissociate upon entering prophase and disappear entirely during the late prophase and metaphase periods of mitosis, then begin to reappear during telophase and form dense organelles during the G1 phase (43). Human RNase III was only translated to and remained in the nucleoli during S phase suggesting that RNase III may have specific functions in nucleoli during S phase. The mechanisms that might regulate nucleolar localization are unknown, but certainly cycles of phosphorylation and dephosphorylation and/or other post-translational modifications may be involved. In any event, the identification of this protein provides an exciting opportunity to better understand human RNA processing.

To evaluate the function of human RNase III, inhibition of expression of the protein with antisense oligonucleotides was performed. The levels of 32 S and 12 S pre-rRNAs were increased after the RNase III was inhibited, suggesting that the protein plays a role in pre-rRNA processing. At least two pathways exist for processing human pre-rRNA (44). In one pathway, human pre-rRNA processing is thought to involve cleavage of 45 S pre-rRNA into 30 S and 32 S fragments (Fig. 6A). The 32 S RNA product of cleavage of 45 S pre-rRNA contains 5.8 S rRNA, ITS2, and 28 S rRNA. Its cleavage results in 12 S pre-rRNA and 28 S rRNA products. The 12 S pre-rRNA is further cleaved to 5.8 S rRNA. Inhibition of RNase III resulted in substantial accumulation of 12 S pre-rRNA, less pronounced accumulation of 32 S pre-rRNA and no accumulation of 45 S pre-rRNA. Thus there is no evidence that this human RNase III is required for the initial cleavage in processing of 45 S pre-rRNA. Rather it appears to be required for the processing of 12 S pre-rRNA. It may also be involved in the processing of 32 S pre-rRNA, but we cannot exclude the possibility that the accumulation of 32 S pre-rRNA is secondary to the inhibition of processing of 12 S pre-rRNA. Therefore, the principle site of cleavage induced by human RNase III observed in these experiments is in the 5.8S-ITS2 region of pre-rRNA. This is distinctly different from the cleavage sites reported for yeast RNase III (RNT1) (23).

Depletion of RNase III resulted in dramatic accumulation of the primary pre-rRNA transcript, 35S RNA, in yeast. Its cleavage sites have been mapped to the 5’- and 3’ETS regions of yeast pre-rRNA. Clearly, this human RNase III appears to be involved in cleavage at a different site in pre-rRNA and a different step in the processing of pre-rRNA relative to yeast. However, additional in vitro cleavage experiments are required to confirm differences in cleavage specificity. Further studies may reveal other members of the human RNase III family involved in different steps in the processing of pre-rRNA. Alternatively, new methods of cellular regulation of this RNase III that result in altered cleavage sites and the ability to participate in other steps in the processing of pre-rRNA may be discovered. Additionally, this enzyme might have other function such as being involved in processing mRNA (2) or small molecular weight nuclear RNAs and small molecular weight nucleolar RNAs (12–14, 18, 19). Our unpublished data showed that after RNase III expression was inhibited by antisense oligonucleotide, ISIS 25691, the level of a DNA-binding protein, TAXREB67, mRNA increased (45). Fig. 5A also shows that after treatment of ISIS 25691, a fragment of RNase III mRNA accumulated, suggesting that RNase III may be involved in mRNA processing, i.e., the inhibition of RNase III may result in
the failure to fully degrade RNase III RNA alter initial cleavage by RNase III.

After treatment with ISIS 25691 for 24–48 h, approximately 10–50% of the cells were dead and detached. In our experiment, we only analyzed attached cells. Attached cells contained relatively high levels of RNase III protein. This might be due to a longer half-life of RNase III protein in these cells which might be in different phases of cell cycle than others. Because the total RNAs analyzed were also only from attached cells, it is likely that the effects on 32 S and 12 S pre-rRNAs accumulation were much more substantial than shown in Figs. 3B and 6. Our kinetic studies showed that RNase III protein declined slightly earlier than the pre-rRNAs accumulated after antisense treatment. In contrast, cell death occurred somewhat later and became pronounced only at the 48-h time point suggesting that the accumulation of the pre-rRNAs may have been lethal. Of course, our studies do not exclude the possibility that human RNase III might have other functions that might be necessary for cell survival.

Despite reducing RNase III mRNA to nearly undetectable levels, we typically observed that the maximal loss of RNase III protein was only approximately 80%. This may be due to an artifact related to the cell death observed and sampling problems deriving from the fact that we evaluated only adherent cells. Alternatively, it is possible that this observation may be biologically relevant and suggest that the protein half-life may be subject to regulation. Clearly, much more work is required to discriminate between these possibilities.

Finally these studies provide a strategy for the functional analysis of newly discovered mammalian genes. When a series of antisense oligonucleotides is designed and tested for reduction of target mRNA levels, varying degrees of reduction of the target mRNA and protein are typically observed. The specificity of each oligonucleotide can be gauged by simultaneous measurement of its effect on several non-targeted mRNAs, and only specific oligonucleotide inhibitors need be considered further. A suspected function of the target gene can be confirmed by an analysis of biochemical events immediately following antisense reduction of target mRNA and protein levels to varying degrees by a series of different oligonucleotides. Rigorous quantitative correlation of the rank order potency of the different oligonucleotides with changes in the downstream biochemical event is strong evidence that the target is functionally related to the event. While gene knockouts can be used to identify essential genes and elucidate biochemical functions in lower organisms, there is no similarly accessible genetic technique in mammalian cells. Knockout mice require significant resources to construct and allow the organism to adjust for the absent gene product during development. In contrast, antisense inhibition of the target mRNA causes a relatively sudden and short-term inhibition of transcription of the target gene and thus provides direct evidence of the function of the gene product in the context of a normal adult phenotype.

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REFERENCES

1. Robertson, H. D. (1990) Methods Enzymol. 181, 189–202
2. Court, D. (1993) in Control of Messenger RNA Stability Belasco, J., Rewold, G., pp. 71–116, Academic Press, Inc., New York
3. Kharrat, A., Macias, M. J., Gibson, T. J., Nilges, M., Pastore, A. (1995) EMBO J. 14, 3572–3584
4. Filippov, V., Solovev, V., Filipova, M., and Gill, S. S. (2000) Gene (Amst.) 245, 213–221
5. Nashimoto, H., and Uchida, H. (1985) Mol. Gen. Genet. 201, 25–29
6. Nicholson, A. W. (1996) Prog. Nucleic Acids Res. Mol. Biol. 52, 1–65
7. Dungan, J. J. (1976) J. Biol. Chem. 251, 3807–3814
8. Zhang, K., and Nicholson, A. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13437–13441
9. Chenfreau, G., Buckle, M., and Jacquier, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3142–3147
10. Arriaino, C. M. (1993) World J. Microbiol. Biotechnol. 9, 421–432
11. Santos, J. M., Drider, D., Marujo, P. E., Lopez, P., and Arraiano, C. M. (1997) EMBO J. 16, 5304–5308
12. Robertson, H. D., and Guthrie, C. (1994) Annu. Rev. Genet. 28, 1–26
13. Staley, J. P., and Guthrie, C. (1998) Cell 92, 315–326
14. Chenfreau, G., Rotondo, G., Legrain, P., and Jacquier, A. (1998) EMBO J. 17, 3726–3737
15. Qu, L. H., Henras, A., Lu, Y. J., Zhou, H., Zhu, W. X., Zhu, Y. Q., Zhao, J., Henry, Y., Caiuzeroux-Ferrer, M., and Bachellerie, J. P. (1999) Mol. Cell. Biol. 19, 1144–1158
16. Chenfreau, G., Legrain, P., and Jacquier, A. (1998) J. Mol. Biol. 284, 975–988
17. Tollervey, D. (1994) Science 263, 1056–1057
18. Tollervey, D., and Kiss, T. (1997) Curr. Opin. Cell Biol. 9, 337–342
19. Elela, S. A., Igel, H., and Ares, M., Jr. (1996) Cell 85, 115–124
20. Kufel, J., Dichtl, B., and Tollervey, D. (1999) RNA 5, 909–917
21. Gegenheimer, U., Watson, N., and Apirion, D. (1977) J. Biol. Chem. 252, 3064–3073
22. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor, NY
23. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Cell 30, 887–896
24. Johnson, R. T., Downes, C. S., and Meyn, R. E. (1993) in The Cell Cycle: A Practical Approach (Pantzer, P., and Brooks, R., eds) pp. 1–24, IRL Press, New York
25. McKay, R. A., Miraglia, L. J., Cummins, L. L., Owens, S. R., Sasmor, H., and Dean, N. M. (1999) J. Biol. Chem. 274, 1715–1722
26. Kingston, R. E. (1997) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 4.2.3–4.2.5, John Wiley & Sons Inc., New York
27. Kosak, M. (1989) J. Cell Biol. 108, 229–241
28. Bram, R. J., Young, R. A., and Steitz, J. A. (1980) Cell 19, 383–401
29. Gonzalez, I. L., Chambers, C., Gorski, J. L., Stambolian, D., Schmickel, R. D., and Sylvester, J. E. (1990) J. Mol. Biol. 212, 27–35
30. Nazar, R. N., Site, T. O., and Busch, H. (1976) Biochemistry 15, 505–508
31. Kozak, M., and Apirion, D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 849–853
32. Marsh, P. E., Ahnn, J., and Inouye, M. (1985) Nucleic Acids Res. 13, 4677–4685
33. Xu, H. P. (1990) J. Biol. Chem. 265, 5304
34. Inoue, Y., Sugimoto, A., and Yamamoto, M. (1991) EMBO J. 10, 221–226
35. Wang, W., and Becker, D. H. (1997) J. Bacteriol. 179, 7379–7385
36. Fu, X. D. (1995) RNA 1, 663–680
37. Robertson, H. D., Webster, R. E., and Zinder, N. D. (1968) J. Biol. Chem. 243, 82–91
38. Anastassova-Kristeva, M. (1977) J. Cell Sci. 25, 103–110
39. Hadjiolova, K. Y., Nicoloso, M., Mazan, S., Hadjiolov, A. A., and Bachellerie, J. P. (1993) Eur. J. Biochem. 212, 211–215
40. Tsujimoto, A., Nyuoya, H., Morita, T., Sato, T., and Shimotohno, K. (1991) J. Virol. 65, 1420–1426