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Rapid Nucleolytic Degradation of the Small Cytoplasmic Y RNAs during Apoptosis*

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We have investigated the fate of the RNA components of small ribonucleoprotein particles in apoptotic cells. We show that the cytoplasmic Ro ribonucleoprotein-associated Y RNAs are specifically and rapidly degraded during apoptosis via a caspase-dependent mechanism. This is the first study describing the selective degradation of a specific class of small structural RNA molecules in apoptotic cells. Cleavage and subsequent truncation of Y RNAs was observed upon exposure of cells to a variety of apoptotic stimuli and were found to be inhibited by Bcl-2, zinc, and several caspase inhibitors. These results indicate that apoptotic degradation of Y RNAs is dependent on caspase activation, which suggests that the nucleolytic activity responsible for hY RNA degradation is activated downstream of the caspase cascade. The Y RNA degradation products remain bound by the Ro60 protein and in part also by the La protein, the only two proteins known to be stably associated with intact Ro ribonucleoprotein particles. The size of the Y RNA degradation products is consistent with the protection from degradation of the most highly conserved region of the Y RNAs by the bound Ro60 and La proteins. Our results indicate that the rapid abrogation of the yet unknown function of Y RNAs might be an early step in the systemic deactivation of the dying cell.

Apoptosis is a form of cell death characterized by distinct morphological and biochemical alterations. Morphologically, apoptotic cells are characterized by chromatin condensation, cell shrinkage, fragmentation of the nucleus, and partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) (1). During the last 5 years, many of the molecules that participate in the biochemical pathway mediating apoptosis have been identified. A major role in this pathway is played by caspases, cysteine proteases with aspartic acid substrate specificity (2). Proteins cleaved by caspases appear to be structural proteins essential for maintaining nuclear and cytoplasmic architecture and enzymes essential for repair of damaged cell components (reviewed in Ref. 3). A prominent nuclear event during apoptosis is internucleosomal cleavage of DNA, recognized as a "DNA ladder" on conventional agarose gel electrophoresis (4). The endonuclease activity responsible for apoptotic degradation of chromosomal DNA has recently been identified (5). The activity depends on two interacting proteins, one of which contains the endonuclease activity (caspase-activated deoxyribonuclease (CAD)[1]), which is retained in the cytoplasm in an inactive form due to its association with the second protein (inhibitor of CAD). Caspase activation in apoptotic cells leads to cleavage of the inhibitor of CAD, thereby releasing active CAD resulting in DNA fragmentation in the nuclei (5, 6).

Much less is known about cleavage and degradation of RNA in apoptotic cells. An increased rate of mRNA turnover has been suggested (7, 8) as well as mitochondrial 16 S ribosomal RNA degradation (9), but no nuclease associated with specific RNA cleavage has been described. Although an increasing number of protein components of ribonucleoprotein particles (RNPs) have been reported to be modified during apoptosis, such as the U1-70K protein, which is a component of the U1 snRNP (10), the 72-kDa component of the signal recognition particle (11), and the La protein, which is associated with several RNPs including the Ro RNPs,[2] no data have been published on the fate of the RNA components of these particles during apoptosis. Therefore, we decided to examine the effects of apoptosis on the cytoplasmic Y RNAs and 7SL RNA, the RNA components of the Ro RNPs and the signal recognition particle, respectively.

Ro RNPs are a class of small cytoplasmic RNA-protein complexes of unknown function, which are present in cells of all species studied to date (reviewed in Ref. 12). In human cells, Ro RNPs consist of one of four small RNA molecules, termed hY1, hY3, hY4, and hY5 (13). All four human Y RNAs have been sequenced (14–16) and found to consist of 112, 101, 93, and 84 nucleotides, respectively, although some heterogeneity at their 3′-ends has been observed. The Y RNAs, which are transcribed by RNA polymerase III (13), are characterized by a conserved stem structure formed by extensive base pairing between the evolutionarily conserved 5′- and 3′-ends. In addition to Y RNAs, Ro RNPs contain at least two different proteins: the La protein and the 60-kDa Ro protein (Ro60), whereas the association of a third protein, the 52-kDa Ro protein (Ro52), is still a matter of debate (17–20). The La protein binds to the oligouridylate stretch at the 3′-end of the Y RNAs, whereas Ro60 interacts with the most highly conserved part of the stem structure (21, 22).

In this study, we observed an extensive, rapid, and selective

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[1] The abbreviations used are: CAD, caspase-activated DNase; RNP, ribonucleoprotein particle; sn, small nuclear.

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nucleolytic degradation of small cytoplasmic RNAs, the Y RNAs, during apoptosis. This phenomenon was observed upon exposure of the cells to multiple apoptotic stimuli, and yRNA degradation appeared to be inhibited by the apoptosis inhibitors Bcl-2 and zinc, as well as by the caspase inhibitors Ac-YVAD-CMK, Z-DEVD-FMK, and Z-IETD-FMK. The results of co-immunoprecipitation experiments and size determination of the apoptotic Y RNA degradation products suggest that the most divergent regions of the Y RNAs are degraded and that the association of Ro60 with the conserved regions is not disrupted, whereas the association with La is partially lost.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat cells, with Bcl-2 (Jurkat/Bcl-2) or without Bcl-2 (Jurkat/Neo) overexpression, kindly provided by Dr. J. Reed (the Burnham Institute, La Jolla, CA) (23), were grown in RPMI (Life Technologies, Inc.) medium supplemented with 10% heat-inactivated fetal calf serum, 200 μg/ml G418 (Life Technologies, Inc.), 1 μM β-mercaptoethanol, 1 mM sodium pyruvate, and penicillin and streptomycin. Mouse WR19L cells expressing human Fas (24) were grown in RPMI (Life Technologies, Inc.) medium supplemented with 10% heat-inactivated fetal calf serum, 200 μg/ml G418 (Life Technologies, Inc.), 1 μM β-mercaptoethanol, 1 mM sodium pyruvate, and penicillin and streptomycin. HeLa cells and HEp-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and penicillin and streptomycin. Cells were cultured in 5% CO_2 at 37 °C.

Apoptotic RNA Isolation and Preparation of Apoptotic Cell Extracts—Jurkat/Bcl-2 and Jurkat/Neo cells and WR19L cells expressing human Fas (24) were treated with an anti-Fas monoclonal antibody 7C11, a kind gift of Dr. M. Robertson (Indiana University, Bloomington, IN), and cells were incubated at 37 °C for the indicated time periods prior to harvesting. HeLa cells were treated with 10 μM actinomycin D and HEp-2 cells with 10 μg/ml anisomycin. Total RNA was isolated by Trizol RNA reagent (Life Technologies, Inc.), according to the instructions of the manufacturer. In parallel, cell extracts were prepared by lysis in Nonidet P-40 lysis buffer (25 mM Tris, pH 7.5, 100 mM KCl, 0.25 mM dithioerythritol, 10 mM $MgCl_2$, 1% Nonidet P-40, protease inhibitor mixture from Roche Molecular Biochemicals) for 30 min on ice. After centrifugation for 15 min at 12,000 $\times$ *g*, supernatants were analyzed by Western blotting. Monolayer cells were trypsinized, washed with phosphate-buffered saline, and treated as above. For experiments utilizing caspase inhibitors, Jurkat cells were cultured in the presence of either 2% Me_2SO, 2 mM $ZnSO_4$, 2 or 20 μM Ac-YVAD-CMK (caspase-1 inhibitor 2, Calbiochem), 2 or 20 μM Z-DEVD-FMK (caspase-3 inhibitor 2, Calbiochem), 2 or 20 μM Z-IETD-FMK (caspase-8 inhibitor 2, Calbiochem) and 2 or 20 μM Z-LEHD-FMK (caspase-9 inhibitor 1, Calbiochem). Subsequently, apoptosis was induced by the addition of anti-Fas monoclonal antibody followed by harvesting after incubations as indicated and lysis as described above.

Northern Blot Analysis—RNA was size-fractionated on 10% denaturing polyacrylamide gels and transferred to Hybond N^+ filters by electroblotting at 3 V/cm in 0.025 M phosphate, pH 6.5, for 2 h. Hybridizations were performed overnight at 65 °C in 6× SSC, 5× Denhardt's solution, and 100 μg/ml sheared, denatured herring sperm DNA with a mixture of ^{32}P-labeled antisense RNA transcripts of the four hY RNAs, antisense 7SL RNA (25), kindly provided by Dr. K. Strub (University of Geneva, Switzerland), or antisense U1 snRNA. Following hybridization, filters were washed twice at 65 °C in 0.2× SSC, 0.1% SDS and were subjected to autoradiography.

In Vitro Transcription—Transcription of antisense hY1, hY3, hY4, hY5, 7SL, and U1 RNA was mainly performed as described (26). To obtain antisense RNAs, pTZ19-hY1, hY3, hY4, hY5, SP64–7SL, and pGEM-U1 RNA were linearized with *Eco*RI. *In vitro* transcription was performed with T7 RNA polymerase (antisense hY1, hY3, hY4, and hY5 RNA) or SP6 RNA polymerase (antisense U1 and 7SL RNA). Transcription and 3′-end labeling of $tRNA^{His}$ was performed as described previously (27).

Western Blot Analysis—Cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis (10%) and blotted onto nitrocellulose filter. After blocking the filters in wash buffer (5% skim milk, phosphate-buffered saline, 0.1% Nonidet P-40) for 1 h at room temperature, filters were incubated with patient serum H42 (anti-U1-70K) at a dilution of 1:5000 in wash buffer for 1 h at room temperature. After washing three times for 15 min, binding of antibodies was visualized by incubation with peroxidase-conjugated rabbit anti-human antibodies (Dako) followed by chemiluminescence detection.

Metabolic Labeling—Jurkat/Bcl-2 and Jurkat/Neo cells were incubated at a density of 2×10^6 cells/ml in labeling medium (RPMI without phosphate (ICN), 2 mM GlutaMAX (Life Technologies, Inc.), 5% dialyzed fetal calf serum 1 mM sodium pyruvate, 10 mM Hepes (pH 7.4), and penicillin and streptomycin). ^{32}P-labeled orthophosphate was added at a concentration of 33 μCi/ml. After incubating the cells at 37 °C for 18 h, an equal volume of RPMI (Life Technologies, Inc.) medium supplemented with 10% heat-inactivated fetal calf serum, 200 μg/ml G418 (Life Technologies, Inc.), 1 μM β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM Hepes (pH 7.4), and penicillin and streptomycin was added. Cells were treated with an anti-Fas monoclonal antibody 7C11 and incubated at 37 °C. RNA was isolated either immediately or after incubation for 1, 2, 3, 4, 6, or 8 h. For protein analysis, radiolabeled cells were solubilized in Nonidet P-40 lysis buffer at the indicated time points. Cell lysates were incubated on ice for 30 min, followed by centrifugation for 15 min at 4 °C at 12,000 $\times$ *g*.

Immunoprecipitation—To immunoprecipitate either unlabeled or radiolabeled RNA, protein A-agarose beads were incubated with rabbit anti-mouse antibodies (Dako) for at least 1 h in IPP_{500} (10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05% Nonidet P-40). After washing three times with IPP_{500}, the beads were incubated with an anti-Ro60 monoclonal antibody (2G10), an anti-La monoclonal antibody (SW5), or an anti-U1-A monoclonal antibody (9A9) by rotation for at least 1 h in IPP_{500}. Incubation was followed by washing twice with IPP_{500} and twice with TKED (10 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM dithioerythritol, 1 mM EDTA, 0.05% Nonidet P-40). After rotating the coated beads with the extracts in TKED for 2 h at 4 °C, the beads were washed three times with TKED. RNA was isolated by phenol/chloroform (1:1) extraction and was precipitated by adding 4 volumes of ethanol and analyzed by 10% denaturing polyacrylamide gel electrophoresis. Radiolabeled RNA was subjected to autoradiography, whereas unlabeled RNA was analyzed by Northern blot hybridization.

RESULTS

hY RNAs Are Specifically Cleaved Early during Apoptosis—To study the effects of apoptosis on the hY RNAs and 7SL RNA, we used two stably transfected Jurkat cell lines, one overexpressing the apoptosis inhibitor Bcl-2 (Jurkat/Bcl-2) and the second a transfection vector control line (Jurkat/Neo). To induce apoptosis, the cells were treated with a monoclonal antibody reactive with Fas (7C11). Previous studies have demonstrated that these antibodies very effectively induce apoptosis in Jurkat cells (11, 28).[2] Cells were harvested either immediately or at the indicated time points after anti-Fas addition. Total RNA was isolated from cell extracts and analyzed by Northern blot hybridization using ^{32}P-labeled antisense hY1, hY3, hY4, hY5, and 7SL RNA probes. The induction of apoptosis was monitored by the analysis of U1-70K protein cleavage, which leads to the appearance of a characteristic 40-kDa product. Cleavage of U1-70K, which is one of the prototypical proteins known to be cleaved during apoptosis (10), was visualized by immunoblotting of cell extracts using a patient serum reactive with the U1-70K protein. Analysis of the hY RNAs revealed that during early stages of apoptosis, these RNAs were efficiently degraded in anti-Fas-treated Jurkat/Neo cells (Fig. 1*A*). Degradation products were already detectable 1.5 h after anti-Fas addition (*lane 13*), whereas a gradual decrease in the amount of intact hY RNAs was evident, with the majority of the hY RNAs being degraded within 4 h after anti-Fas addition (compare *lane 18* with *lane 11*). Although all four hY RNAs were degraded upon induction of apoptosis, slight differences were observed in the rate of degradation. The rate of degradation appeared to be related to the size of the hY RNA; hY1 was degraded most quickly. In contrast, no degradation of 7SL RNA was observed in these cells (Fig. 1*A*). The selectivity of degradation of hY RNAs in apoptotic cells was further substantiated by the lack of detectable degradation of several other small RNAs, including U snRNAs, tRNAs, and 5 S rRNA (data not shown).

Degradation of hY RNAs was inhibited in Jurkat cells overexpressing Bcl-2, because a slight decrease of the amount of hY

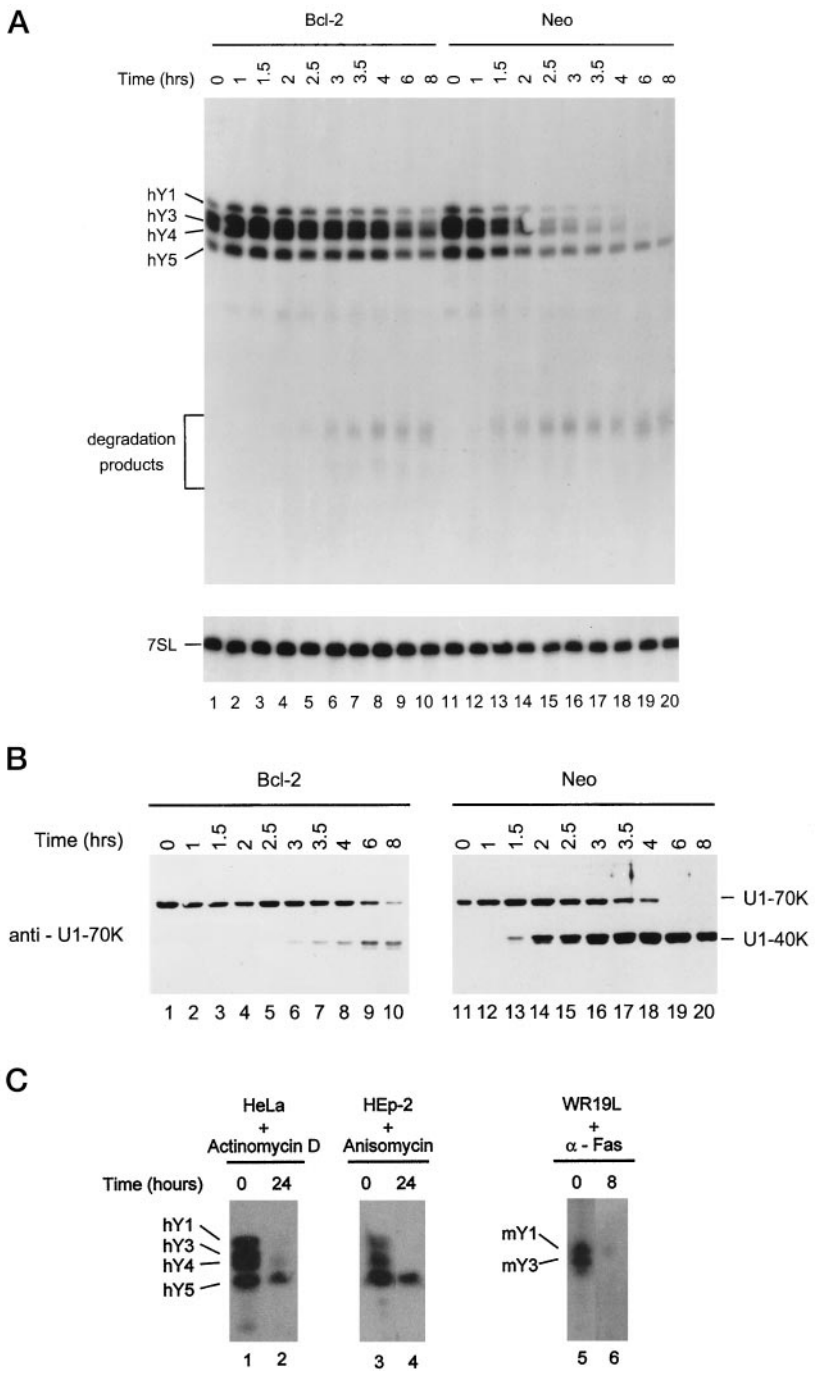

FIG. 1. **Degradation of hY RNAs during apoptosis.** Jurkat/Bcl-2 (*Bcl-2*) and Jurkat/Neo (*Neo*) cells were treated with the anti-Fas monoclonal antibody 7C11 for various time periods (indicated above the lanes in hours). *A,* total RNA isolated from Jurkat/Bcl-2 (*lanes 1–10*) and Jurkat/Neo cells (*lanes 11–20*) was analyzed by Northern blot hybridization using a mixture of hY RNA probes (*upper panel*) and a 7SL RNA probe (*lower panel*). The positions of the hY RNAs, their degradation products, and 7SL RNA are indicated. *B,* control for the induction of apoptosis monitored by U1-70K cleavage, which is visualized by Western blot analysis using a polyclonal anti-U1-70K serum. The positions of the U1-70K protein and its apoptotic cleavage product (U1–40K) are indicated. *C,* degradation of Y RNAs occurs in cells triggered by the apoptotic stimuli and in cells derived from other species. Human HeLa cells treated with actinomycin D (10 μM) (*lanes 1* and *2*), HEp-2 cells treated with anisomycin (10 μg/ml) (*lanes 3* and *4*), and mouse WR19L cells expressing human Fas (13) treated with anti-Fas monoclonal antibody (*lanes 5* and *6*) were harvested after incubation for various time periods (indicated above the lanes). Total RNA was isolated and analyzed by Northern blot hybridization using a mixture of hY RNA probes. The positions of the hY RNAs are indicated. Note that in mouse cells, only mY1 and mY3 are present.

RNAs was only detectable 6 h after anti-Fas addition, whereas degradation products did not appear during the first 3 h (*lanes 1–10*). The delayed degradation of hY RNAs in Jurkat/Bcl-2 cells in comparison with the Jurkat/Neo cells reflected the different efficiencies of apoptosis induction in these cells, which was monitored by cleavage of the U1-70K protein (Fig. 1*B*) and by flow cytometry of annexin-V stained cells (results not shown).[2] The kinetics of hY RNA degradation appeared to be very similar to that of U1-70K cleavage, which is known to be mediated by caspase-3, suggesting that hY RNA degradation might also be dependent on caspase activation.

To determine whether degradation of hY RNAs also occurs when apoptosis is triggered by other stimuli, and in cells derived from other species, we analyzed apoptotic cell extracts derived from human HeLa cells treated with actinomycin D (10 μM) (data not shown) or HEp-2 cells treated with anisomycin (10 μg/ml). Also, apoptotic cell extracts, derived from mouse WR19L cells expressing human Fas (24) and treated with anti-Fas monoclonal antibody, were analyzed. Total RNA was isolated and analysis of Y RNAs by Northern blot hybridization revealed that Y RNA degradation was observed in all cells tested (Fig. 1*C*). Induction of apoptosis in all cells was confirmed by cleavage of the U1-70K protein.

Although the normal turnover rate of hY RNAs is known to be relatively low, a reduction of the hY RNA levels in apoptotic cells might in principle be due to either inhibition of RNA polymerase III transcription or to an increased rate of hY RNA degradation. To exclude the possibility that the reduction in hY RNA levels was caused by abrogation of their synthesis and to investigate the association of the Ro RNP with hY RNAs in apoptotic cells, RNA in Jurkat/Bcl-2 and Jurkat/Neo cells was radiolabeled by culturing the cells in the presence of ^{32}P-or-

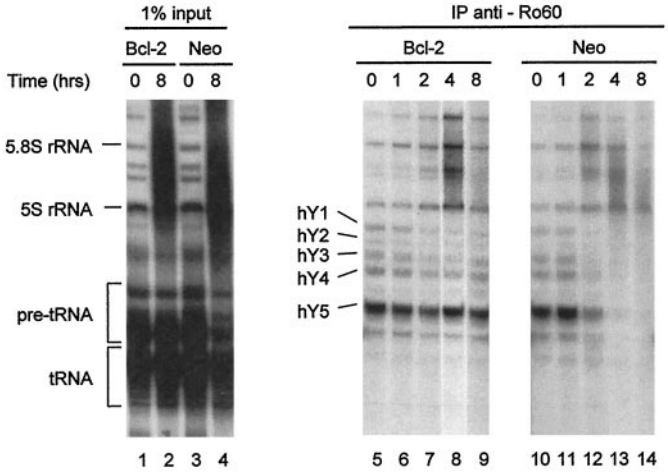

FIG. 2. **Stability of hY RNAs in Jurkat cells and Ro RNP association of hY RNAs during apoptosis.** Jurkat/Bcl-2 (*Bcl-2*) and Jurkat/Neo (*Neo*) cells were cultured in the presence of ^{32}P-labeled orthophosphate (in phosphate-free medium) for 18 h at 37 °C. After replacing the labeling medium with complete medium, apoptosis was induced by anti-Fas addition, and cell extracts were prepared either immediately or after incubations for the indicated time periods (above the lanes). Total RNA isolated from the 0 and 8 h extracts (*lanes 1–4*) and hY RNAs isolated from Jurkat/Bcl-2 extracts (*lanes 5–9*) and Jurkat/Neo extracts (*lanes 10–14*) by immunoprecipitation with an anti-Ro60 monoclonal antibody (2G10) were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The positions of pre-tRNA, tRNA, 5 S rRNA, 5.8 S rRNA, and the hY RNAs are indicated. The amount of RNA electrophoresed in *lanes 1–4* corresponds to 1% of the amount of cell extracts used for hY RNA isolation.

thophosphate for 18 h. After labeling, apoptosis was induced by anti-Fas addition in complete medium, *i.e.* in the presence of an excess of unlabeled phosphate. Cells were lysed either immediately or after incubation for the indicated time periods, and induction of apoptosis was monitored by cleavage of the U1-70K protein (data not shown). The results for the Jurkat/Bcl-2 cells illustrate the low turnover rate of hY RNAs (Fig. 2, *lanes 5–11*). Even at 8 h after replacement of radiolabeled phosphate with unlabeled phosphate, little or no decrease of radiolabeled hY RNAs was observed, which is indicative of the relatively long half-life of these molecules. This result confirms that the observed decrease in hY RNAs levels during apoptosis is due to a strongly increased degradation rate rather than the abrogation of hY RNA synthesis.

Because an immunoprecipitation step was required to isolate the low abundance hY RNAs from the total pool of radiolabeled RNAs, this experiment also provided information on the Ro RNP association of radiolabeled hY RNAs. Immunoprecipitation was performed with an anti-Ro60 monoclonal antibody (2G10), and co-precipitated RNAs were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The results in Fig. 2 show that co-immunoprecipitation of hY RNAs from apoptotic Jurkat/Neo cell extracts decreased during the first hours after anti-Fas addition and was hardly detectable at the 4 h time point (*lanes 10–14*). The decrease in hY RNA precipitation by anti-Ro60 antibodies is likely to be indeed caused by hY RNA degradation rather than by disruption of the interaction between hY RNAs and Ro60, because the analysis of Ro60 from apoptotic cells by Western blotting did not reveal detectable changes, such as proteolytic cleavage (Ref. 28 and data not shown). Moreover, the RNA binding capacity of Ro60 was not abolished in apoptotic Jurkat cells, as demonstrated by the co-precipitation of hY RNA degradation products (see below). It should also be noted that the disappearance of full-length hY RNAs isolated by immunoprecipitation from radiolabeled cell extracts resembles the decrease of hY RNA signals obtained by Northern blot analysis of total RNA (Fig. 1). Taken together, these results demonstrate that the disappearance of hY RNAs during apoptosis is indeed due to degradation and that hY RNAs remain in association with the Ro RNP in apoptotic cells until or even after the degradation process has been initiated.

The differences in RNA ranging in size approximately from 5 to 5.8 S rRNA between total radiolabeled RNA isolated either immediately after anti-Fas addition (Fig. 2, *lanes 1* and *3*) or following 8 h of incubation (*lanes 2* and *4*) might be due to apoptotic degradation of ribosomal RNA and/or mRNA (7–9), resulting in higher background signals. Note that due to the low abundance of radiolabeled hY RNAs, relatively high background signals of much more abundant RNAs, such as 5 and 5.8 S rRNA (Fig. 2, *lanes 1* and *3*), were observed among the immunoprecipitated RNAs (*lanes 5–14*).

Effect of Caspase Inhibitors on Degradation of hY RNAs—Caspases are not only involved in the activation of apoptotic proteases; also, caspase-dependent activation of a deoxyribonuclease has recently been reported (5). To study the role of caspases in the activation of the nuclease activity responsible for hY RNA degradation during apoptosis, Jurkat cells were cultured in the presence of several caspase inhibitors, including zinc sulfate (29), the caspase-1 inhibitor Ac-YVAD-CMK, the caspase-3 inhibitor Z-DEVD-FMK, the caspase-8 inhibitor Z-IETD-FMK, or the caspase-9 inhibitor Z-LEHD-FMK for 1 h prior to and during anti-Fas treatment. Cells were harvested either immediately or 4 or 8 h after anti-Fas addition. Total RNA was isolated and analyzed by Northern blotting using hY RNA probes and a 7SL RNA probe as a control. Fig. 3*A* demonstrates that hY RNA degradation was completely inhibited in Jurkat cells cultured in the presence of zinc sulfate (Fig. 3*A, lanes 4–6*). hY RNA degradation was also clearly inhibited by the addition of Ac-YVAD-CMK, Z-DEVD-FMK and Z-IETD-FMK (Fig. 3*B, lanes 3–8*) in comparison with the control incubation with 2% Me_2SO (Fig. 3*B, lane 2*). In contrast, the caspase-9 inhibitor Z-LEHD-FMK only poorly affected hYRNA degradation (Fig. 3*B, lanes 9–10*). As expected, the addition of these inhibitors had no effect on 7SL RNA signals (Fig. 3, *lower panels*). As a control for the inhibitory activity of the tetrapeptide inhibitors, the cell extracts were also analyzed for U1-70K cleavage, which is known to be sensitive to Ac-DEVD-CHO (10, 28, 30). Cleavage of the U1-70K protein was indeed inhibited by $ZnSO_4$ and the caspase-1, caspase-3, and caspase-8 inhibitors and to a lesser extent by the caspase-9 inhibitor (data not shown). These results demonstrate that the apoptotic degradation of hY RNAs is dependent on caspase activation.

The Ro60 and La Proteins Remain Associated with the Apoptotic Degradation Products of hY RNAs—The results described above indicate that hY RNAs remain associated with the Ro60 protein until or possibly even during the degradation process. Therefore, it was possible that at least some of the degradation products were still bound by either the Ro60 or the La protein, the two proteins that are directly bound to the hY RNAs in Ro RNP complexes. To investigate the potential interaction of these proteins with the apoptotic degradation products hY RNAs, immunoprecipitation experiments were performed with monoclonal anti-Ro60 (2G10) and anti-La (SW5) antibodies. Cell extracts were prepared at various time points after the addition of anti-Fas antibody and RNA was analyzed by Northern blot hybridization either directly isolated from cell extracts or following immunoprecipitation. Fig. 4*A* shows RNA isolated from cell extracts, corresponding with 10% of the cell extracts used for immunoprecipitation. As is shown in Fig. 4, *B* and *C,* both the full-length hY RNAs and at least part of the degradation products were co-immunoprecipitated with anti-

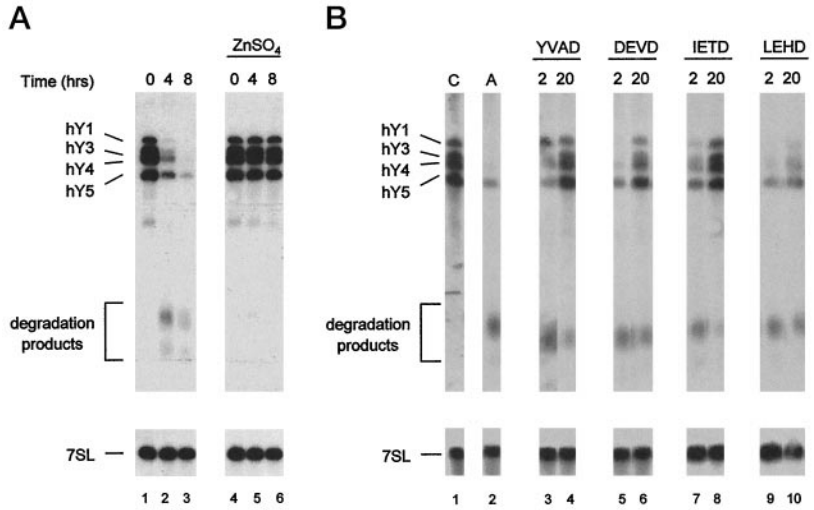

FIG. 3. **Effect of caspase inhibitors on hY RNA degradation.** *A,* Jurkat/Neo cells were cultured for 1 h in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of 2 mM $ZnSO_4$. Subsequently, the cells were incubated with the anti-Fas monoclonal antibody 7C11 for 0, 4, or 8 h, followed by total RNA isolation and Northern blot analysis by hybridization with a mixture of hY RNA probes (*upper panels*) and with a 7SL probe (*lower panels*). The positions of the hY RNAs, their degradation products, and 7SL RNA are indicated. *B,* Jurkat/Neo cells were cultured for 1 h in the presence of 2% Me_2SO (*lanes 1* and *2*), 2 μM (*lanes 3, 5, 7,* and *9*) or 20 μM (*lanes 4, 6, 8,* and *10*) Ac-YVAD-CMK (*lanes 3* and *4*), Z-DEVD-FMK (*lanes 5* and *6*), Z-IETD-FMK (*lanes 7* and *8*), or Z-LEHD-FMK (*lanes 9* and *10*), which were all dissolved in Me_2SO (final concentration in culture, 2% Me_2SO). Subsequently, the anti-Fas monoclonal antibody 7C11 was added, and the cells were incubated for 4 h. The RNA analysis was performed as described in the legend to Fig. 3*A*. Material from nonapoptotic control cells (*C*) is analyzed in *lane 1,* whereas *lane 2* contains apoptotic (*A*) material (from cells cultured for 4 h in the presence of anti-Fas antibody). The positions of the hY RNAs, their degradation products, and 7SL RNA (*lower panels*) are indicated.

Ro60 (Fig. 4*B*) and anti-La (Fig. 4*C*) antibodies. This strongly suggests that both proteins remain associated with the hY RNAs during the nucleolytic process and thus with the respective binding site containing degradation products of the hY RNAs. The anti-Ro60 antibody co-precipitated the degradation products much more efficiently than the anti-La antibody, which might indicate that although both antibodies seem to co-precipitate the same set of degradation products, the La binding site might be partially lost. A control immunoprecipitation was performed with a monoclonal antibody (9A9) to the U1A protein, a protein specifically associated with the U1 snRNP. As expected, U1 snRNA was not co-precipitated with anti-Ro60 and anti-La antibodies, and no hY RNAs were co-precipitated with the anti-U1A antibodies (Fig. 4*D*). In contrast, U1 snRNA was efficiently precipitated by the anti-U1A antibodies, substantiating the specificity of the immunoprecipitations.

Determination of the Length of the Apoptotic Degradation Products of hY RNAs—To determine the length of the apoptotic degradation products of the hY RNAs, ^{32}P-labeled Jurkat/Neo cell extracts were used to isolate hY RNA degradation products by immunoprecipitation with anti-Ro60 monoclonal antibody 2G10. An anti-Ro60 antibody was used because the degradation products were efficiently co-precipitated by this antibody and because the pattern of degradation products precipitated by this antibody was indistinguishable from the pattern observed in total RNA (which was more evident when a longer exposure of Fig. 4*A* was compared with Fig. 4*B*; data not shown). Precipitated RNAs were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography (Fig. 5*A*). As RNA size marker 3′-end-labeled $tRNA^{His}$ (27), either partially digested under denaturing conditions by RNase T1 (*lane 1*) or denatured at 94 °C in the presence of 10 mM $MgCl_2$ (*lane 2*), was used. Consistent with the results shown in Figs. 1–4, 2 h after anti-Fas addition, a decrease in the amount of full-length hY RNAs was observed, with the simultaneous appearance of the degradation products (*lane 5*). The result showed that the apoptotic hY RNA degradation products range in size from 22 to 36 nucleotides. The heterogeneity in size of these fragments is at least in part due to (i) the fact that the fragments are derived from four distinct RNAs (hY1, hY3, hY4, and hY5), (ii) the known 3′-end heterogeneity of native hY RNAs, and (iii) the fact that both 5′-end and 3′-end fragments of these RNAs will be present in the immunoprecipitate, taking into account that the Ro60 binding site is composed of a hybrid of these fragments.

The apoptotic degradation products of hY RNAs were more efficiently precipitated by anti-Ro60 antibodies than by anti-La antibodies (Fig. 4, *B* and *C*), indicating that the La association and/or the La binding site might be partially lost. To study this in more detail, we also isolated hY RNA degradation products by immunoprecipitation with anti-La monoclonal antibody SW5 and compared these products with the degradation products immunoprecipitated with anti-Ro60 antibodies by denaturing gel electrophoresis (Fig. 5*B*). The results showed that in addition to the differences in efficiency of precipitation, the patterns of co-precipitated molecules were different. Most notably, the smallest apoptotic hY RNA degradation products immunoprecipitated with anti-Ro60 antibodies, ranging in size from 22 to 25 nucleotides, were not immunoprecipitated by anti-La antibodies (Fig. 5*B*), which strongly suggests that these degradation products have lost the 3′ oligouridine stretch. Although all the other bands (27–36 nucleotides), with the exception of the 31-nucleotide-long molecule(s), were detectable in the anti-La selected material, their relative intensities showed some variation, which is of course not surprising if we take into account that these bands also contain molecules corresponding to the 5′-end of the hY RNAs, which are not present in the anti-La precipitate when they are annealed with 3′-fragments from which the La binding site has been removed.

DISCUSSION

Previous studies have demonstrated that the Ro RNP-associated Ro proteins and La are clustered in two distinct populations of blebs at the surface of apoptotic cells (31). The Ro52 protein is present in small apoptotic blebs together with fragmented endoplasmic reticulum and ribosomes. The larger blebs, called apoptotic bodies, contain the La protein, the Ro60 protein, small nuclear ribonucleoproteins, and nucleosomal

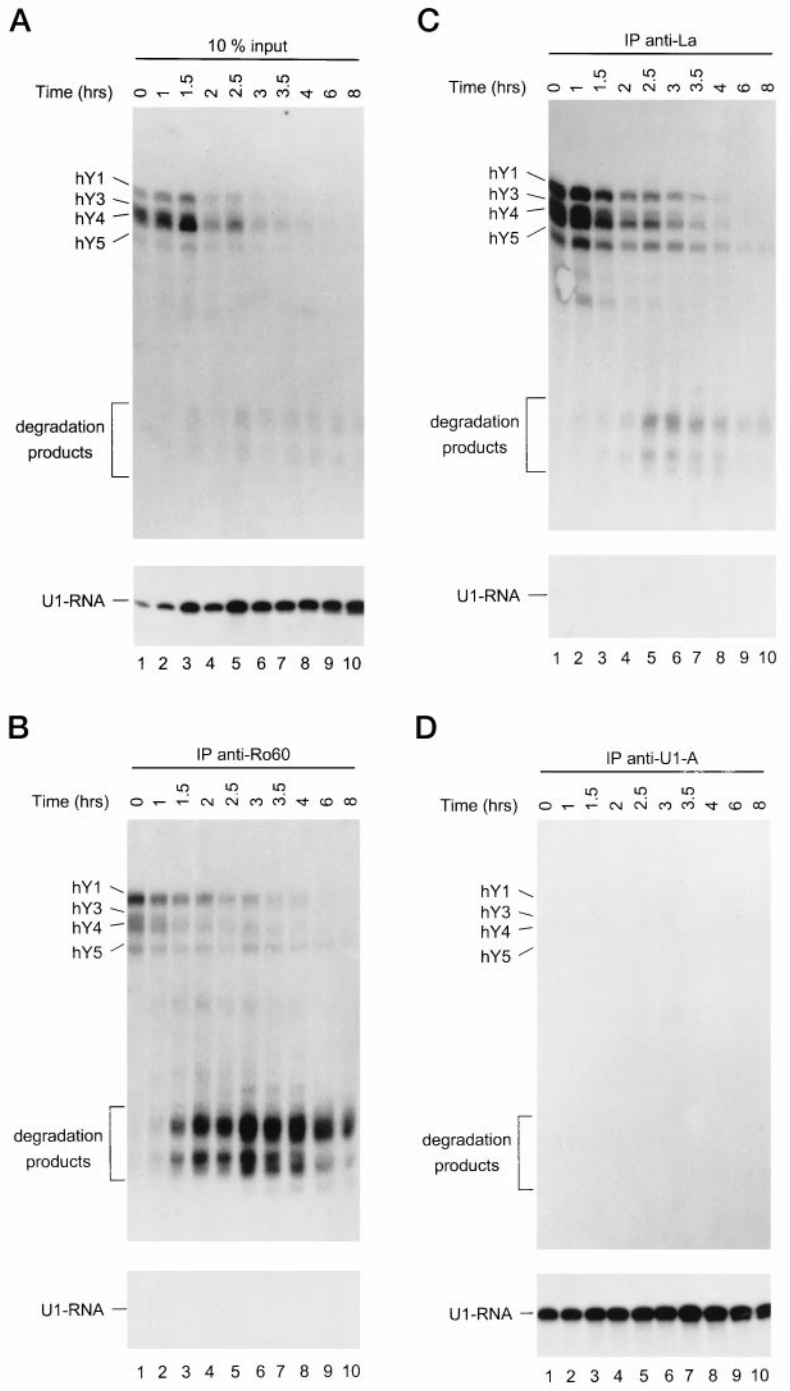

FIG. 4. **The Ro60 and La proteins are associated with the apoptotic hY RNA degradation products.** Jurkat/Neo cells were treated with anti-Fas antibody, and cell extracts were prepared at the indicated time points. RNPs were isolated by immunoprecipitation with anti-Ro60 (2G10), anti-La (SW5), or anti-U1A (9A9) monoclonal antibodies. RNAs isolated from the immunoprecipitates were analyzed by Northern blot hybridization using a mixture of hY RNA probes (*upper panels*) or a U1 snRNA probe (*lower panels*). *A,* total RNA isolated from cell extracts, corresponding to 10% of extracts used for immunoprecipitations (note that due to an electrophoresis artifact the amount of total RNA in *lane 1* is less than 10% of input material); RNA co-immunoprecipitated by anti-Ro60 (2G10) (*B*), anti-La (SW5) (*C*), and anti-U1A (9A9) (*D*). The positions of the hY RNAs, their degradation products, and U1 RNA are indicated.

DNA (31, 32). A more detailed analysis of both Ro proteins as present in apoptotic cells by Western blotting did not show obvious changes, such as a proteolytic cleavage (33). However, we observed recently that the La protein is rapidly dephosphorylated during apoptosis, and in addition, a subset of the La molecules is cleaved.[2] In this study, we have demonstrated that the RNA components of Ro RNPs, the Y RNAs, are efficiently degraded early during apoptosis, whereas several other small RNAs, including 7SL RNA, U snRNAs, tRNAs, and 5 S rRNA, are not detectably affected. Degradation of Y RNAs was observed in a variety of cell types and after induction of apoptosis by a variety of stimuli. Apoptotic Y RNA degradation was inhibited by the caspase-1 inhibitor Ac-YVAD-CMK, the caspase-3 inhibitor Z-DEVD-FMK, and the caspase-8 inhibitor Z-IETD-FMK after anti-Fas induced apoptosis, strongly suggesting that this process is dependent on caspase activation. Activation of effector caspases, such as caspase-3 and related proteases, can be mediated by the activation of initiator caspases, such as caspase-8 and -9. Caspase-8 is activated by signals from death receptors at the cell surface (*e.g.* the Fas receptor) (34), whereas caspase-9 is activated by Apaf1 in cells undergoing drug-induced apoptosis (34, 35). Y RNA degradation is observed in anti-Fas-treated cells as well as in cells treated with actinomycin D and anisomycin, indicating that activation of the nuclease involved can be induced by both pathways, consistent with the activation of the nucleolytic activity by a general effector caspase. Such a mechanism is supported by the inhibitory effect on hY RNA degradation of the caspase-1, -3, and -8 inhibitors in anti-Fas-treated cells. These results demonstrate that Fas-induced hY RNA degradation is

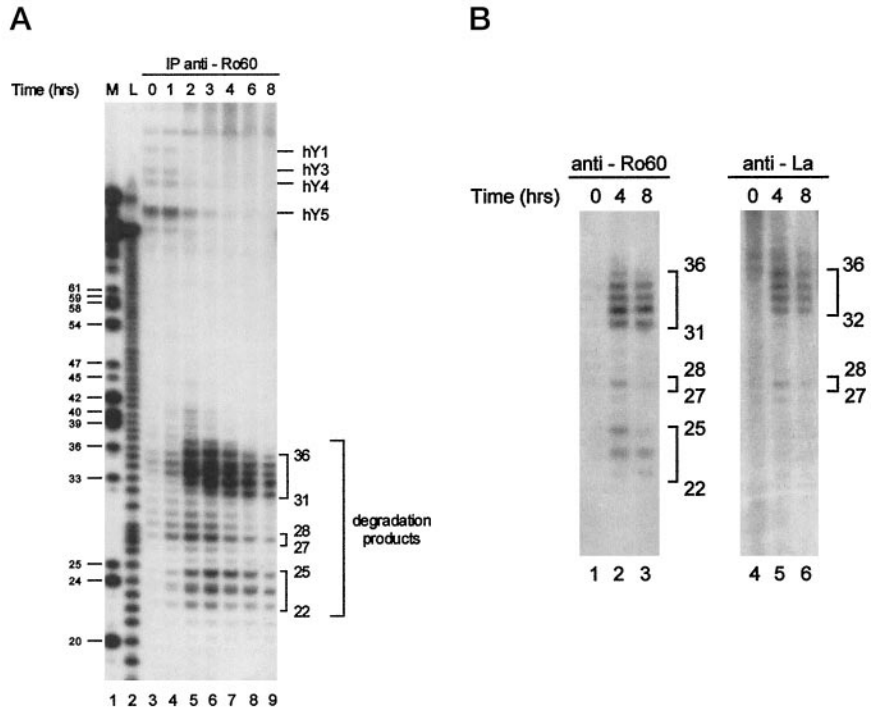

FIG. 5. **Determination of the length of the hY RNA degradation products.** Jurkat/Neo cells were labeled by culturing in the presence of ^{32}P-orthophosphate in phosphate-free medium for 18 h at 37 °C. After the addition of an equal volume of phosphate-containing medium, apoptosis was induced by anti-Fas addition. *A,* hY RNAs were isolated from cell extracts prepared at the time points indicated above the lanes by immunoprecipitation with an anti-Ro60 monoclonal antibody (2G10). RNA was isolated from the immunoprecipitates and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. As the RNA size marker, 3′-end-labeled tRNA^{His} (4), either partially digested under denaturing conditions by RNase T1 (*M, lane 1*) or partially hydrolyzed at 94 °C in the presence of 10 mM $MgCl_2$ (*L, lane 2*), was electrophoresed in parallel. RNA marker lengths are indicated on the *left,* and the positions of the hY RNAs and their degradation products are indicated on the *right*. *B,* hY RNA degradation products were isolated from cell extracts prepared at the time points indicated above the lanes by immunoprecipitation with an anti-Ro60 monoclonal antibody (2G10) (*lanes 1–3*) or with an anti-La monoclonal antibody (SW5) (*lanes 4–6*). RNA was isolated from the immunoprecipitates and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. The length of the hY RNA degradation products (see *A*) is indicated on the *right*.

dependent on caspase-8 activation and suggest that a subsequently activated effector caspase, such as, *e.g.* caspase-1 or caspase-3, is involved in activation of the nucleolytic activity. As expected, hardly any inhibition of hY RNA degradation was observed by the caspase-9 inhibitor in anti-Fas induced apoptotic cells, in agreement with the fact that caspase-9 does not play a major role in this pathway (34). It should be stressed that the data obtained with the tetrapeptide caspase inhibitors should be interpreted with care, because the specificity of the inhibitors is not absolute (36, 37). For instance, the slight inhibition observed with the caspase-9 inhibitor might be due to cross-inhibition of another caspase. We conclude that apoptotic hY RNA degradation is caspase-dependent and that the ribonuclease(s) involved is most likely activated by the action of one or more effector caspases. The size and protein binding characteristics of the most stable apoptotic degradation products suggest that the central parts of the Y RNAs are cleaved by an endonuclease activity and that these regions are further degraded up to the region that is protected by the stably bound Ro60 and La proteins.

At present, little is known about degradation or cleavage of RNA in apoptotic cells. An increased rate of mRNA turnover has been reported (7, 8) as well as mitochondrial ribosomal RNA degradation (9), but so far, no nuclease activity associated with specific RNA degradation has been described. The best characterized nuclease that is specifically activated in apoptotic cells is the DNase involved in internucleosomal cleavage of chromatin, resulting in the typical DNA ladder. In nonapoptotic cells, this CAD is located in the cytoplasm in a complex with the inhibitor of CAD. Upon induction of apoptosis, the inhibitor is cleaved by caspase-3, thereby releasing active CAD, which then is able to enter the nucleus (5). Human homologues for CAD and the inhibitor of CAD, which were initially characterized after purification from mouse cells, have been described recently and were designated CPAN and DFF45, respectively (38, 39). CAD and CPAN have no significant homology to known nuclease protein families and may, therefore, represent a new class of endonucleases (38). Because apoptotic degradation of Y RNAs is also dependent on caspase activation, it is tempting to speculate that the (endo)nuclease involved in apoptotic Y RNA degradation might be a member of this new class of endonucleases.

The secondary structure of Y RNAs is characterized by a pyrimidine-rich internal loop and a long stem structure formed by extensive base pairing between the highly conserved 5′- and 3′-ends (Fig. 6). Binding of the Ro60 protein to the Y RNAs has been extensively studied (21, 22, 40). The central part of the conserved stem structure, highlighted in Fig. 6 by the *boxed areas,* appeares to be essential for binding the Ro60 protein (40). The binding of the Ro60 protein, possibly in combination with the La protein, protected the RNA against pancreatic ribonuclease digestion (22). Because the apoptotic degradation products of the hY RNAs are at least in part associated with Ro60 and the La protein, which binds to the oligouridylate stretch at the 3′-end of the RNAs, it is likely that protection against the RNA degrading activity by these proteins also occurs in apoptotic cells.

The length of the Ro60-associated apoptotic degradation products, as determined in a denaturing gel system, ranges from approximately 22 to 36 nucleotides (Fig. 5*A*), whereas the length of the degradation products that remain associated with the La protein ranges from 27 to 36 nucleotides. The size

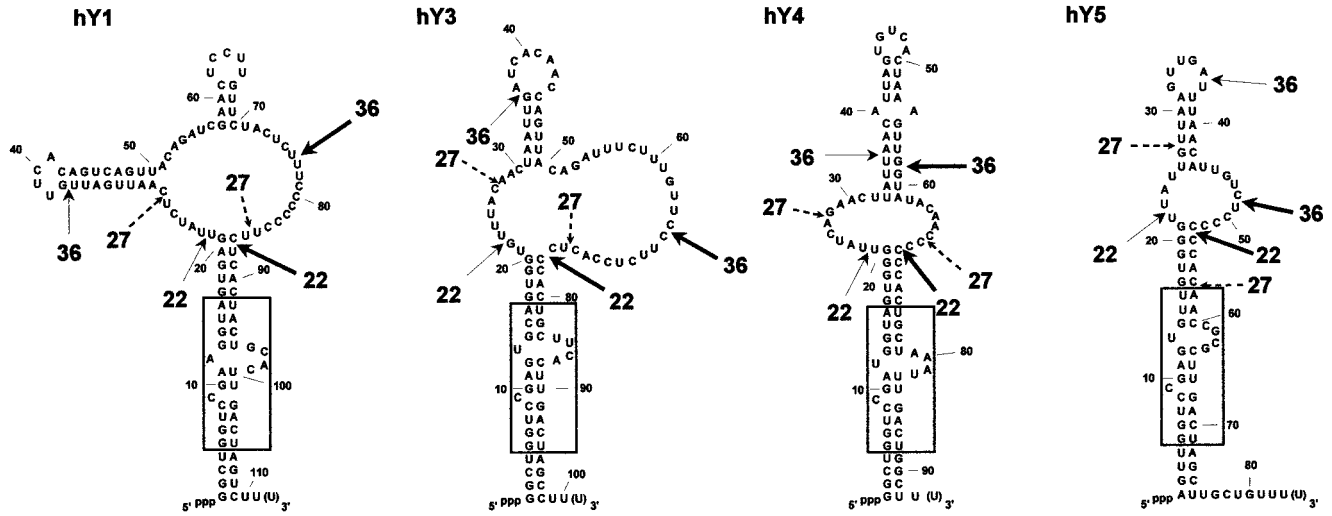

FIG. 6. **Secondary structures of the hY RNAs and possible apoptotic degradation products.** The secondary structures shown are according to Van Gelder *et al*. (43). The regions that are involved in Ro60 binding, derived from data published by Green *et al*. (40), are *boxed*. The regions harboring the potential termini of the smallest (22 nucleotides) and largest (36 nucleotides) degradation products associated with Ro60 are indicated with *arrows*. *Thin arrows* border the regions for termini of 5′-end fragments, whereas *bold arrows* border the regions for termini of 3′-end fragments of the hY RNAs. Note that the positions of the *bold arrows* bordering the 22 nucleotides 3′-terminal fragments, which represent fragments that are not associated with the La protein, are based on the assumption that the single-stranded nucleotides at the 3′-ends are removed. *Dotted arrows* mark terminal fragments of 27 nucleotides, *i.e.* the minimal length of the fragments that co-immunoprecipitate with the anti-La monoclonal antibody.

heterogeneity is most likely, at least in part, explained by the hY RNA heterogeneity, the 3′-end heterogeneity of individual hY RNAs, and the presence of fragments derived from both the 5′- and 3′-ends of the RNAs. In addition, fragments derived from the 5′-end of the RNAs might still contain a triphosphate entity at their 5′-end, whereas, also, the phosphorylation state at the termini of the fragments generated during apoptosis is unknown. The negative charge of terminal phosphates is known to influence the mobility of relatively small molecules in denaturing polyacrylamide gels. This may explain the "half-nucleotide" migration shifts of the most prominent hY RNA degradation products with respect to the marker lane (Fig. 5*A*), and this makes it impossible to designate the size of the degradation products to one-nucleotide resolution. Most of the hY RNA degradation products will be derived from hY5 RNA, because in nonapoptotic cells, this RNA is most efficiently labeled, consistent with the relative abundance of hY RNAs in human cells (41). A schematic representation of the hY RNAs and the regions in which the termini of the degradation products map are shown in Fig. 6. From these results, we conclude that the degradation products are not simply the result of a single cleavage event. Most likely, an initial endonucleolytic cleavage occurs in the central part of the hY RNAs, possibly in the pyrimidine-rich internal loop, subsequently followed by either exonuclease digestion starting at the initial cleavage site or additional endonucleolytic cleavages. The slight decrease in the sizes of the degradation products observed between 2 and 6 h after the induction of apoptosis suggests the involvement of an exonucleolytic activity. The exclusive association of Ro60 and not La with the group of smaller degradation products (22–25 nucleotides) strongly suggests that also from the 3′-end of the hY RNAs, nucleotides may be removed in apoptotic cells, resulting in disruption of the La binding site.

Many caspase substrates are proteins involved in important cellular processes, such as cell cycle regulation, signaling, DNA repair, cell homeostasis, and cell survival, suggesting that proteolytic disabling of certain key proteins directly contributes to the irreversibility of the apoptotic process. In view of their rapid, specific, and efficient degradation during apoptosis, this might also be true for Y RNAs. Unfortunately, the function of Ro RNPs is still unknown, although recently, a role for Y RNAs in the translational control of ribosomal protein mRNAs, and possibly other 5′-terminal oligopyrimidine-containing mRNAs, has been suggested (42). The possible involvement in the systemic disassembly of the dying cell might give new clues to elucidate the function of Ro RNPs.

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