We have identified a cellular target for proteasomal endonuclease activity. Thus, 20 S proteasomes interact with the 3′-untranslated region of certain cytoplasmic mRNAs in vivo, and 20 S proteasomes isolated from Friend leukemia virus-infected mouse spleen cells were found to be associated with a mRNA fragment showing great homology to the 3′-untranslated region of tumor necrosis factor-β mRNA that contains AUUUA sequences. We furthermore demonstrate that 20 S proteasomes destabilize oligoribonucleotides corresponding to the 3′-untranslated region of tumor necrosis factor-α, creating a specific cleavage pattern. The cleavage reaction is accelerated with increasing number of AUUUA motifs, and major cleavage sites are localized at the 5′ side of the A residues. These results strongly suggest that 20 S proteasomes could be involved in the destabilization of cytokine mRNAs such as tumor necrosis factor-α mRNAs and other short-lived mRNAs containing AUUUA sequences.

During the last 2 years, the proteasome has emerged at the forefront of modern cell biology. It is currently evident that the proteasome plays a key role in multiple events. Thus, it is involved in the regulation of transcription, antigen presentation, and the degradation of damaged and misfolded proteins, and it drives the cell cycle (1). In addition, there is evidence for the involvement of the proteasome in post-transcriptional control of gene expression (2, 3).

The 20 S proteasome is a highly organized multimeric protein complex forming a cylindrical structure that has been detected in all eukaryotic cell systems investigated so far as well as in bacteria (1). The proteasome cylinder consists of a stack of two α discs and two central β rings, each of which is composed of seven subunits with molecular masses between 19 and 35 kDa. Two-dimensional gel electrophoresis revealed up to 20 individual proteins with the number varying between cells and species. The β rings of the 20 S proteasome harbor at least five endopeptidase activities that are differently regulated in an ATP-dependent fashion by two copies of a 19 S protein complex, which can associate with the α discs of the 20 S core proteasome, forming the 26 S proteasome with a molecular mass of about 2000 kDa. This complex processes and degrades ubiquitinated proteins (1).

In addition, proteasomes have been reported to contain about 0.0016–0.2% low molecular mass RNAs. The RNA content depends on the origin of the proteasomes and decreases with increasing proteasome purity (4, 5). These RNAs are heterogeneous in size but migrate most frequently in a molecular size range of 70–200 nucleotides (5). Partial sequence analysis of proteasomal RNAs has revealed that no particular species of RNA is specifically associated with the proteasomes. These proteasomal RNAs were suggested to represent contaminations of purified proteasomes (4) or residual substrate fragments of a specific proteasomal RNase activity (2). The latter hypothesis is more attractive because it has been shown that endonuclease activity is associated with subunits i and l in the α discs of proteasomes, indicating the existence of a close and specific relationship between proteasomes and RNA (6). In this context, 20 S proteasomes (prosome) were detected as factors that control translation of Friend leukemia virus-infected mouse erythroblasts by transient association with free mRNAs (5, 7). How proteasomes select senescent free mRNAs for degradation remains unknown. Work on mRNA decay has shown that sequence elements such as the 3′-untranslated region (UTR) regulate the degradation of different eukaryotic messengers. The best studied are the AUUUA-rich motifs within the 3′-UTR of short-lived mRNAs named ARE. The mechanisms by which these mRNAs are targeted for rapid degradation and the factors that are involved in this association with ARE have not been definitively characterized (8).

In the present paper, we provide evidence in vivo and in vitro that 20 S proteasomes associate with 3′-UTR mRNA fragments containing AUUUA motifs. In addition, we first demonstrate that AUUUA-rich elements are degraded by 20 S proteasome-associated RNase activity at specific cleavage sites.

**EXPERIMENTAL PROCEDURES**

**Definition of TBK Buffers—** Buffer TBK X consists of 20 mM Tris-HCl, pH 7.4, x mM KCl, 2.5 mM MgCl₂, and 7 mM 2-mercaptoethanol, where x is 100, 240, or 550.

**Cell Fractionation Procedure and Isolation of Proteasomes—** 20 S proteasomes were extracted from Friend leukemia virus-infected mouse spleen or calf liver by differential centrifugations (9) and highly purified by fast protein liquid chromatography (FPLC) as described previously (2).

**Protein Gel Electrophoresis—** Proteins were precipitated with 10% metaphosphoric acid. The precipitates were dissolved in 100 mM Tris-HCl, pH 7.4, 8 M urea, 0.1% SDS, heated at 100 °C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis (5%).
trichloroacetic acid (final concentration) or 2 volumes of ethanol. Electrophoresis of proteins was on one-dimensional SDS-polyacrylamide gels (according to Ref. 10). Molecular mass markers were: phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14 kDa).

Proteasome RNA Extraction and RNA Electrophoresis—Proteasomes from sucrose gradient fractions were precipitated with 2 volumes of ethanol and sedimented by centrifugation in a HB-4 rotor Sorvall (20 min, 9000 rpm, 4 °C). Pellets were resuspended in Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, pH 7.5, and 0.5% sodium laurylsulfosuccinate). Then 0.2 mg/ml of proteinase K was added, and incubation was carried out for 1.5 h at 37 °C. RNA was extracted from this suspension with chloroform/phenol (11) and precipitated with 2.5 volumes ethanol and 0.3 M sodium acetate. Such purified RNA was labeled at the 3' end with cytidine 3'-5'[γ-32P]ATP in a reaction catalyzed by T4 RNA ligase (12). Labeled proteasome RNA was separated on a 1% sequencing gel as described previously (5).

Assay of Proteasome RNase Activity and RNA Sequencing—The quantity of proteasomes was determined by the Bio-Rad assay. The synthetic oligomers were purchased from Eurogentec (Belgium). RNA oligomers were labeled at the 5' end with [γ-32P]ATP (Amersham) or [γ-32P]ATP and T4 polynucleotide kinase (Boehringer Mannheim) according to the manufacturer’s instructions and then incubated with 50 μg of proteasomes for 30 min at 37 °C in TBK-240. The digests were extracted from enzymatic assays with chloroform/phenol (11) and run on a 16% sequencing gel with 1× TBE running buffer for 2 h at 50 watts. The ladder was obtained by alkaline hydrolysis of RNA oligomers (13).

[^3H]Poly(U) Hybridization—Identification of poly(A)^+ sequences in mRNAs was by hybridization with [^3H]poly(U) as described (14).

Cloning and Sequencing Analysis of the cDNAs of Proteasome-associated RNAs—This procedure was described in detail (15). Briefly, proteasomes RNA were extracted from proteasomes as described above and polyadenylated (16). cDNAs were synthesized using oligo(dT) primers as described (17). After synthesis of the DNA double strand and ligation with a BglII linker, the proteasome DNA was inserted in a pGEM plasmid and transformed into Escherichia coli DH1 strain (18). Finally, the plasmid was isolated and submitted to restriction endonuclease analysis (19). Plasmid DNA was sequenced using the dyeode procedure (20).

RESULTS

Detection of Proteasomes Associated with the 3' Ends of Cellular mRNAs—When a post-mitochondrial supernatant of mouse erythroblasts was analyzed by sucrose gradient centrifugation, proteasomes probed with a monoclonal antibody sedimented exclusively in subribosomal fractions smaller than the ribosomal subunits 40 S (Fig. 1). In this sedimentation range, proteasomes comigrated with a heterogeneous population of poly(A)^+ mRNAs, which was detected by [^3H]poly(U) hybridization (Fig. 1). As previously reported (21), these mRNAs are not engaged in translation and consist of a pool of senescent mRNAs and of mRNAs that are stored for future translation. If proteasomes are involved in the degradation of senescent mRNAs containing AREs, they should interact with their 3’-UTR near the poly(A)^+ tail. Moreover, if the proteasome 20 S complex that is bound to 3’-UTR partially overlaps with the poly(A)^+ sequence, incubation with RNase A and RNase T1 should release proteasome/UTR/poly(A)^+ complexes because poly(A)^+ is not hydrolyzed under these conditions. To investigate this idea more closely, the particles between 10 and 30 S were pooled and analyzed by further centrifugation on 10–50% sucrose gradients in Tris buffer containing 100 mM KCl (TBK 100). These particles sedimented with 2 major maxima of absorbance in the range of 13 S, a protein complex of unknown function and others observed earlier (5, 22), and 20 S, consisting mainly of proteasomes, and one smaller in the range of about 30 S (Fig. 2A). Poly(A)^+ mRNAs sedimented between 10 and 30 S with major distribution in zones corre-

sponding to the maxima of absorbance (Fig. 2A). The presence of proteasomes was identified by their typical protein pattern (right panels of Fig. 2).

After incubation with RNase T1 and RNase A, [^3H]poly(U) hybridization revealed a sharp peak of labeled poly(A)^+ containing particles in the range of 10 S (23, 24) and a smaller population that sedimented in the range of 20 S, together with proteasomes (Fig. 2B). However, when RNase-treated particles were analyzed by centrifugation through sucrose gradients containing TBK 350, much less poly(A)^+ containing fragments sedimented with 20 S proteasomes (Fig. 2C). From these experiments, we concluded that proteasomes retained poly(A)^+ containing mRNA fragments at 100 mM KCl, while they dissociated from the proteasome complex at 350 mM KCl. This is in good agreement with results we have published earlier showing that proteasomes dissociate completely from mRNAs at 820.
Fig. 2. Sedimentation profile of subribosomal particles after RNase treatment. Particles of 12 gradients sedimenting between 30 and 10 S (Fig. 1, fractions 10–18) were pooled and concentrated by sedimentation (Beckmann rotor T60, 50,000 rpm, 4 °C, 19 h). Solid line, absorbance at 254 nm; line with filled squares, [3H]poly(U) hybridization (see Fig. 1); P, proteasomes. Right panels, the protein pattern of the proteasome. A, pellets were resuspended in TBK 100 and analyzed by sedimentation through 10–50% (w/w) sucrose gradients (Beckmann rotor SW40, 36,000 rpm, 4 °C, 18 h). B, pellets resuspended in TBK 100 were incubated for 30 min at 37 °C with RNase A (5 μg/ml) and RNase T1 (20 units/ml) and immediately analyzed by sedimentation through 10–50% (w/w) sucrose gradients in TBK 100 (Beckmann rotor SW40, 36,000 rpm, 4 °C, 18 h). C, pellets resuspended in TBK 100 were incubated for 30 min at 37 °C with RNase A (5 μg/ml) and RNase T1 (20 units/ml) and immediately analyzed by sedimentation through 10–50% (w/w) sucrose gradients in TBK 350 (Beckmann rotor SW40, 36,000 rpm, 4 °C, 18 h). D, schematic presentation of the RNase protection experiments.
AREs Are Targets for 20 S Proteasome Endonuclease Activity

Identification of a Proteasome-associated RNA Fragment with ARE That Maps in the 3′-UTR of TNFβ mRNAs—Another strong argument for a possible association of proteasomes with mRNAs is the presence of different smaller RNA molecules in proteasomes preparations. The length of these RNAs and the RNA content varies from preparation to preparation. Based on these findings, we postulated recently that at least some of these RNA molecules could be residual fragments of the RNA substrates cleaved by the endonuclease activity that is associated with proteasomes (2, 3, 6).

To elucidate this hypothesis, we sequenced some proteasome-associated RNAs. For this approach, 10–30 S particles (Fig. 1) were pooled and further sedimented through 10–50% sucrose gradients in TBK 350. Under these conditions, poly(A)+ mRNAs dissociated completely from 20 S proteasomes (Fig. 3A). Such purified proteasomes were pooled (Fig. 3A, fractions 12 and 13), and the purity of the particles was tested by Laemml polyacrylamide gel electrophoresis. Fig. 3B shows the typical pattern of proteasomal subunits migrating between 20 and 35 kDa with some faint bands of nonproteasomal proteins in the range of 43–68 kDa.

Proteasome-associated RNAs were extracted from these fractions, labeled at the 3′ end with cytidine 3′-5′-[5-32P]triphosphate, and analyzed by RNA gel electrophoresis. Fig. 3C shows typical profiles of proteasome-associated RNAs, which migrate as a heterogeneous population ranging from 60 to 500 nucleotides. Highly purified proteasomes washed with 1% lauroylsarcosyl contain almost one RNA species, which was identified to be tRNA leucyl (3, 25). For sequencing, RNAs were extracted from less purified 20 S fractions. After polyadenylation, cDNAs of proteasome-associated RNAs were synthesized and cloned in a pGEM I transcription vector. Sequencing of eight cDNA clones using dideoxynucleotides revealed quite different sequences (15). We here show sequence number 4 (P4), which contains several AUUUA motifs (Fig. 4A). Further computer analysis revealed that this sequence is 86% homologous with the 3′-UTR of mouse TNFβ mRNA (Fig. 4B).

Proteasomes Destabilize the 3′-UTR of TNFα mRNA—Several cellular proteins acting as trans-factors were reported to recognize AREs and may target, in some way, cytokine mRNAs like TNFα mRNA for degradation. Among these factors that associate specifically with ARE, a 20 S protein complex with unknown protein composition was described to be involved in the destabilization of ARE (26). Because 20 S proteasomes and this 20 S protein complex share common properties and because we have identified an RNA fragment containing AUUUA motifs almost homologous to TNF mRNA in proteasome preparations, we wanted to investigate whether proteasomes could be involved in the degradation of ARE.

In a first series of experiments, we incubated highly purified proteasomes with [γ-32P]ATP-labeled 3′-5′-[5-32P]triphosphate and used in degradation assays. To address this question, different synthetic oligoribonucleotides containing no, two, or four copies of the AUUUUA motif were labeled with [γ-32P]ATP and used in degradation assays. mm KCl (Refs. 5 and 7 and Fig. 3A). These observations suggest that proteasomes interact directly with the poly(A)+ sequence or very nearby in the 3′-UTR of certain mRNAs.
We found that a minimum of two AUUUA motifs is required for the destabilization by proteasomes. We detected only one cleavage site with (AUUUA)\textsubscript{2} oligo-RNA, whereas incubation of (AUUUA)\textsubscript{4} oligo-RNA with proteasomes produced five fragments corresponding to five cleavage sites (Fig. 6). Obviously, preferential cleavage sites are situated at the 5' end of the nucleotide A within a AUUUA motif. Moreover, the densitometric analysis of native oligonucleotides containing two or four motifs after incubation with proteasomes for various times revealed that (AUUUA)\textsubscript{4} oligo-RNA was degraded much faster than the oligo-RNA containing two AUUUA motifs (data not shown). These data correlate well with in vivo experiments (27).

**DISCUSSION**

Despite the identification of several proteins that associate with RNAs containing AUUUA motifs in vitro, none has been definitively linked to ARE-mediated degradation of mRNAs. Most of these proteins migrated on polyacrylamide gels in a range of 30–45 kDa. First identified was a protein with a molecular mass of 44 kDa containing three subunits and named AUBF (adenosine-uridine binding factor (28). Using an in vitro destabilizing system, Brewer (29) identified two polypeptides of 37 and 40 kDa that are implicated in the accel-
eration of degradation of ARE containing messengers. Others described a 32-kDa protein identical to HuR that specifically cross-links to (AUUA)$_4$ and (AUUUUA)$_3$ but not (AUU)$_3$ and (AUUA)$_2$ RNAs in vitro (30). This protein would play an RNA-stabilizing role in the ARE-directed mRNA decay in mammalian cells (31).

On the other hand, a cytoplasmic 20 S protein complex with unknown protein composition was described to be involved in the destabilization of AREs (26). This work was of particular interest because we have recently shown that a similar cytoplasmic structure, the 20 S proteasome, harbors a specific endonuclease activity (2). In addition, the results in this paper clearly demonstrate that proteasomes hydrolyze oligo-RNAs with (AUUA)-rich multimers at specific sites in vitro, which corresponds well with the in vivo observations of other groups. Cellular mRNAs with ARE in their 3'-UTR are expressed transiently with half-lives in the order of 10–30 min (32), whereas β-globin mRNA that contains only one AUUUA motif is very stable with a half-life greater than 17 h (33). Several studies have demonstrated that deletion or replacement of the adenose- and uridine-rich sequence in the 3'-UTR of c-fos mRNA conferred significantly greater stability to the previously unstable messenger (34), whereas the addition of a short DNA sequence from the granulocyte-monocyte colony stimulating factor 3'-UTR into the 3'-UTR of the rabbit β-globin gene destabilized the corresponding naturally stable β-globin mRNA (27). This could explain why globin mRNA with one AUUUA motif was not degraded by proteasome-associated endonuclease activity (2). For accelerated degradation, proteasomes apparently require multiple AUUUA motifs (at least two) that partially overlap. Based on these results, we concluded that proteasomes could be involved in the degradation of ARE. This conclusion correlates well with the following observations: (i) purified proteasomes from Friend leukemia virus-infected mouse spleen cells contain adenose- and uridine-rich RNA fragments with great homology to the 3'-UTR of TNFβ mRNA (Fig. 4); (ii) Arrigo et al. (35) have demonstrated by RNA sequencing that Drosophila proteasomes are associated with RNA fragments that possess extended adenose- and uridine-rich elements; and (iii) finally, our RNase protection experiments confirmed that proteasomes interact specifically with the 3'-end of a nondefined number of cytoplasmic RNAs. However, the binding site is not the poly(A)$^\text{+}$ sequence because proteasome-associated RNA fragments reported so far do not have oligo(A) stretches, and proteasomes associate strongly with TMV-RNA without poly(A)$^\text{+}$ or with adenovirus mRNAs of which the poly(A)$^\text{+}$ tail is blocked by oligo(T) (36). We suspect that proteasomes bind to sequences adjacent or within adenose- and uridine-rich elements close to poly(A)$^\text{+}$ and cleave the 3'-end with the poly(A)$^\text{+}$ sequences of the corresponding messengers. This idea fits well with the hypothesis of other groups that postulate the existence of a specific cytoplasmic endonuclease activity to be involved in post-transcriptional gene silencing (26).

The results suggest that proteasomes could inhibit the translation of certain cytokine mRNAs and in general of other short-lived mRNAs using their ARE as target for degradation. Inappropriate translation of these cellular mRNAs has dramatic effects because their protein products interfere with cell division and cell differentiation. Interestingly, viral mRNAs like tobacco mosaic virus RNA with much less extended adenosine- and uridine-rich motifs was reported to be a good substrate for proteasome-associated RNase activity (2). Based on these observations, selective recognition of proteasome RNA substrate and the association of proteasomes with RNA sequences cannot be restricted to the presence of adenose- and uridine-rich elements alone. In fact, RNAs of different origin like tRNA$^{\text{lys}}$, viral RNAs, or 18 S rRNA that associate with proteasomes suggest the existence of a common secondary structure or box-like sequence. Indeed preliminary sequence comparison of these mRNAs and RNA fragments we found in proteasomes preparations revealed a common nucleotide motif that will be presented in a subsequent paper. Taken together, we concluded that the endonuclease activity associated with the α subunits $\gamma$ and of the 20 S proteasome complex (6) is rather important for cellular events.

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Possible Involvement of Proteasomes (Prosome) in AUUUA-mediated mRNA Decay
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