The endoribonuclease RNase E is believed to initiate the degradation of many mRNAs in Escherichia coli, yet the mechanism by which it recognizes cleavage sites is poorly understood. We have prepared derivatives of the mRNA encoding ribosomal protein S20 which contain a single major RNase E cleavage site at residues 300/301 preceded by variable 5′ extensions. Three of these RNAs are cleaved in vitro with significantly reduced efficiencies relative to the intact S20 mRNA by both crude RNase E and pure Rne protein (endonuclease component of RNase E). In all three substrates as well as in the full-length mRNA the major cleavage site itself remains single-stranded. One such substrate (t84D) contains a 5′-stem-loop structure characterized by three noncanonical A-G pairs. Removal or denaturation of the stem restores efficient cleavage at the major RNase E site. The other two contain single-stranded 5′-termini but apparently lack cleavage sites near the termini. Our data show that sensitivity to RNase E can be influenced by distant structural motifs in the RNA and also suggest a model in which the initial recognition and cleavage of a substrate near its 5′ end facilitates sequential cleavages at more distal sites. The model implies that RNase E contains at least a dimer of the Rne subunit and that the products of the first cleavage are retained by Rne prior to the second cleavage.

Ribonuclease (RNase) E has emerged as the principal endonuclease involved in the turnover of a number of mRNAs and some small RNAs (reviewed in Refs. 1, 2). The features of its substrates which render them susceptible to attack at specific sites have been the subject of considerable investigation. Tomcsányi and Apirion (3) initially proposed that RNase E recognizes a sequence of 10 residues which is substantially conserved within two sites in 9 S RNA and the single site in the ColE1-specified RNA 1. Characterization of further RNase E cleavage sites in a variety of RNAs in vivo and in vitro has demonstrated that there is modest similarity among the primary sequences cleaved (1, 4). Rather, the major feature of an RNase E cleavage site is its tendency toward richness in A and U residues (4–6). The most common site of cleavage is immediately 5′ to an AU dinucleotide; nonetheless, there are numerous exceptions. This feature alone would be insufficient to explain the enzyme’s specificity on most of its substrates. Attempts to correlate the sites of cleavage with experimentally determined secondary structures in substrates have shown that RNase E cleaves single-stranded residues (7, 8). In addition, cleavage sites are usually preceded (e.g. 9 S site “a” (7)) or followed (e.g. T4 gene 32 mRNA (1)) by a stable stem-loop structure. Whether such structures actively facilitate cleavage or simply restrict the number of single-stranded regions available to the enzyme has been debated. Recent experiments using relatively short oligonucleotides based on the sequence of the 5′ end of RNA 1 have demonstrated that such molecules are efficient substrates for RNase E in the absence of secondary structural features (9). These data were also interpreted to indicate that stem-loops can in some cases impede RNase E cleavage; similar conclusions have been drawn for derivatives of the mRNA encoding ribosomal protein S20 (10). Indeed, in this substrate, cleavages at sites in the relatively unstructured 5′ end (56 residues) occur more rapidly than cleavage at the major internal site at residues 300/301 and 301/302 (4).

Other features of an RNA substrate in addition to the immediate environment of a cleavage site may mediate susceptibility to RNase E. The ompA mRNA which is relatively resistant to decay in vivo (11) contains a highly structured stem-loop structure at its 5′-terminus (12, 13). Single-stranded extensions as short as 3 unpaired residues are sufficient to reverse the stabilizing effects of the 5′-stem structure (14). The role of 5′ structures in determining resistance or susceptibility to RNase E has not been investigated systematically in vitro, however.

We have constructed several substrates derived from the mRNA for ribosomal protein S20 with the view of eliminating all but one RNase E site. Several of these modified RNAs proved to be very poor substrates, despite retaining an unaltered cleavage site. We have investigated the structures of these RNAs in order to determine the basis for their resistance to cleavage.

**EXPERIMENTAL PROCEDURES**

Templates and RNAs—Plasmid pGM87 containing the P2 leader, coding sequences, and rho-independent terminator of the gene for S20 in the vector pTZ18U (15) has been described previously (10; see also Fig. 1). Transcription of the S20 mRNA in pGM87 is under control of the T7 promoter. Plasmids pJG175 and pJG194 were constructed using polymerase chain reaction technology as described (16). The forward primers, oligonucleotides 461 (5′-ccgaaattctagctaatgactagCACACAGAAAGACATTATAACGGAATGG) or 444 (5′-ccgaaattctagctaatgactagCACACAGAAAGATCGTGAGG) respectively, contain an EcoRI site and a T7 RNA polymerase promoter in addition to S20 sequences. The lowercase letters denote residues not found in the natural S20 mRNA. The forward primer for pSM160, oligonucleotide 1110 (5′-ttgagatttaagctaatgactagCTATGAAATTCGCAACACGCGTGTG) spans the HindIII site between residues 411–412 (see Fig. 1). After amplification of 10 ng of linearized pGM79 (4) or pOM87, the product was cleaved with EcoRI and HindIII and purified by electrophoresis on a 6% polyacrylamide gel. The appro-
pG98-2, is a derivative of pRC9S (7) in which the T7 RNA polymerase promoter of the latter has been replaced by an SP6 promoter. Messenger-sense RNA was trancribed in the presence of [γ-32P]CTP from plasmids linearized with Dral (4, 10) or either AccI (7) or HindII in the case of pG98-2, as described. Thus t87D denotes the transcript obtained from pGM57 linearized with Dral.

Preparation of Extracts and Assay of RNase E Activity—AS-26 fractions containing an rne-dependent endonuclease activity were prepared as described (4). The final concentration of protein in assays was 0.1 mg/ml for AS-26 fractions prepared from CF881 (19), 0.065 mg/ml for AS-26 fractions from strain GM402 (20), and approximately 0.5 μg/ml for the renatured Rne protein purified as in Ref. 20. Substrate RNAs were renatured by heating in the assay buffer for 2 min at 50°C and 10 min at 37°C followed by chilling (treatment "A"). Alternatively, t84D was boiled in H2O for 120 s prior to chilling and supplementation with assay buffer (treatment "B"), RNase assays were performed at 30°C with 20 mM substrate RNA. Samples taken after different times of incubation were quenched in 90% formamide containing tracking dyes, denatured, and separated on 6% polyacrylamide gels containing 8 M urea in Tris borate-EDTA buffer (4). Products were visualized by autoradiography and quantified with a Molecular Dynamix PhosphorImager. Initial rates of product formation were calculated from first-order plots containing at least six points. Treatment of substrate RNAs with RNase H (1 unit) and a 5–10-fold excess of oligonucleotide 481, 5′-TTTCTGTGTCAGCAGCAG (treatment "C"), was performed as described (4).

RNA Structure Mapping—5′-End-labeled RNAs were prepared by transcription of DNA templates in the presence of 50 μCi of [γ-32P]CTP (Amersham Corp.) and sufficient unlabeled GTP to a final concentration of 50 μM. ATP, CTP, and UTP were present at 0.5 mM. Labeled RNA transcripts were purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and two cycles of precipitation with ethanol in the presence of 2 M ammonium acetate. In one preparation, samples were passed over a Sephacryl S-400 HR MicroSpin™ column (Pharmacia Biotech Inc.) after the first ethanol precipitation. Conditions for RNA renaturement and probing were similar to those used previously (18). Samples containing unlabeled t84D and labeled RNA at 20 mM final concentration were renatured or boiled (treatments A and B above, respectively) and then digested with one of the following enzymes in RNase E assay buffer (4) at 30°C for times ranging from 3 to 10 min. Optimal concentrations of T1 (Pharmacia), CL3 (Boehringer Mannheim), V1 (Pharmacia), and T2 RNAse (a gift of Dr. George Chaconas, University of Western Ontario, London, ON, Canada) were determined empirically and are given in the legend to Fig. 3. PhoII-aceacte was used at 2.5 mM for 5.0 min. Digestions were terminated with the addition of carrier RNA (25 μg), EDTA to 10 mM, SDS to 0.2%, and sodium acetate to 0.25 M and were extracted once with phenol/chloroform/isoamyl alcohol. The RNAs were recovered by ethanol precipitation, dissolved in a buffer containing 90% formamide, denatured by boiling, and analyzed by electrophoresis on sequencing gels. Markers were prepared by heating labeled RNA for 5 min at 100°C in 50 mM sodium carbonate, pH 9 (alkaline ladder), or by digesting labeled RNA supplemented with 3 μg of carrier yeast RNA with 0.8 units of T1 RNase for 6.5 min at 50°C in 50 mM sodium citrate, pH 5.0, containing 7 M urea (G ladder).

RESULTS

Effect of 5′ Deletions on RNase E Cleavage of the mRNA for Ribosomal Protein S20 in Vitro—Previous work showed that sequential 5′ to residue 178 in stem II of the synthetic S20 mRNA transcript t87D (Fig. 1) were dispensable for cleavage at residues 300/301/302 (8). In an effort to reduce the number of cleavage sites and to simplify the analysis of the products and their kinetics of formation, we constructed a template, pGM54, encompassing residues 249–447 of the S20 sequence (numbered as in Fig. 1). Transcription of Dral-cleaved pGM54 would yield a 222-residue run-off RNA (t84D). The first 22 residues in this RNA are derived from sequentes in the vector and the polylinker. Unexpectedly, this RNA was a poor substrate for both crude and purified preparations of RNase E (Fig. 2; see also Table I). The initial rate of formation of 147-residue product from t84D at limiting amounts of crude RNase E activity (from the AS-26 prepared from strain CF881) was reduced 10-fold (treatment A in Table I) relative to the parental substrates, t79D and t87D (Fig. 1 and Refs. 4, 10). These measurements were repeated with more enriched sources of RNase E activity: an AS-26 from strain GM402 which overexpresses the Rne polypeptide and RNase E activity or the electrophoretically purified Rne protein itself (20). In both cases, the rates of cleavage at the RNase E-sensitive site (residues 300/301/302) were considerably reduced relative to the activity on the full-length substrate (Table I).

The inefficiency of cleavage of the 222-residue t84D substrate could be due to interference from the additional 22 transcribed residues derived from the vector. Accordingly, more precise deletions were effected using polymerase chain reaction technology such that a T7 (or SP6) RNA polymerase promoter was inserted into a predetermined location in the S20 template (see “Experimental Procedures”). One such transcript, t194D, would initiate at residue 258 (changing C258 to G) and retain 12 residues at the bottom of stem III (refer to Fig. 1). Transcripts from a second construction, t175D, would initiate at residue G268 (see below), substitute a G residue for residue C269 and cleanly lack any part of stem III. In several assays with different preparations of RNase E, t194D was cleaved at a very slow rate indistinguishable from that of t84D (Fig. 2C; data summarized in Table I, treatment A), whereas t175D was virtually uncleavable (data not shown). A final substrate, t160D, whose 5′ end maps to residue G268, was, however, cleaved relatively efficiently (Table I). The efficiency of cleavage of these substrates appears to depend, therefore, on the position of the 5′ end rather than on the presence or absence of vector sequences.

Competition experiments were performed to test whether RNAs such as t84D or t1194D could function as inhibitors of RNase E activity in the AS-26 extract prepared from CF881. The data in Table II show that t84D in form A (poorly cleaved; see below) is unable to compete with 9 S RNA (7) for cleavage, whereas the more readily cleavable form of t84D, form B (see below), is a modest competitive inhibitor. Likewise, the rate of cleavage of 9 S RNA at a and b sites to yield pre-5 S RNA is not altered appreciably by the presence of a 3-fold molar excess of t194D (data not shown). In contrast, an efficiently cut derivative of the S20 mRNA (t85D; Ref. 10) does compete well with 9 S RNA for cleavage (data not shown). These observations are consistent with t84D and t194D being poor substrates for RNase E rather than efficient inhibitors which sequester the enzyme in an inactive state.

Activation of Poorly Cleaved Substrates—The “silencing” of the RNase E sites at residues 300/301/302 in t194D and t175D but not in t160D and the failure of the former to act as a competitor suggested that some or all of residues 258–288 can inhibit the cleavage process in cis, possibly by forming inhibitory secondary structures. Several experiments were performed to test this possibility. In the first, prior boiling was employed to remove or alter the putative inhibitory structure(s). This treatment activated t84D quite significantly (treatment B in Table I). The rate of cleavage of t84D increased 5-fold with an AS-26 fraction from strain CF881, the crudest fraction assayed. Significantly, there was a 3-fold increase in the rate of cleavage of boiled t84D even with the purified Rne protein. In contrast, none of the other truncated templates could be activated by boiling nor could the full-length substrate (Table I; see also Refs. 4, 8).
In the second approach, oligonucleotide-directed cleavage by RNase H (Ref. 21; Treatment C in Table I) was employed to truncate substrates. Primer extension experiments showed that the resultant 5'-termini map to residues 276–280 (data not shown). The data in Table I show that both t84D and t194D can be activated substantially by such truncation to achieve rates comparable with those observed with the full-length substrate (t87D) treated similarly. The initial rate of cleavage of t84D at residues 300/301/302 by AS-26 fractions increased over 50-fold after prior shortening dependent on oligonucleotide 481 and RNase H (Fig. 2b; Table I). Treatment C also enhanced the activity of the purified Rne protein toward t84D over 35-fold (column III). Similar increases were observed with t194D as a substrate, using either an AS-26 from strain CF881 (Fig. 2d; column I in Table I) or the purified Rneprotein (column III). In the latter case, the absolute rates of cleavage are lower than for t84D, possibly because the RNase H-mediated truncation is incomplete, but the increase induced by treatment C (28-fold) is comparable with that seen with t84D. Omission of RNase H or substitution of a non-complementary oligonucleotide resulted in no activation of cleavage of any of the substrates by purified Rne or by cruder fractions (data not shown). Thus, prior modification including denaturation (t84D) or 5'-truncation is completely capable of reversing the otherwise low rate of cleavage of the substrates t84D and t194D.

Attempts were made to activate t175D using oligonucleotide-targeted RNase H digestion (treatment C). The cleavage of this substrate by RNase H was very inefficient despite a nominal 10-residue complementarity between oligonucleotide 481 and residues 270–279 in t175D (data not shown). Nonetheless, after treatment C the rate of appearance of the 147-residue product from t175D increased to a barely detectable level but still well below that observed with the other substrates (data not shown).

Structure Mapping of t84D—To explore any structural differences among the various substrates, we performed structure mapping experiments by digesting end-labeled RNA substrates with structure-specific ribonucleases. In the case of t84D, we compared the substrate's sensitivity after renaturation (denoted as form A) or boiling (form B). Two important sets of observations emerged. First, residues C298, C299, and G300 immediately 5' to the RNase E cleavage site are equally reactive toward CL3 and T1 after either pretreatment (Fig. 3a, lanes 5–8). Likewise, A301 and U302 3' to the cleavage site are equally reactive toward dimethyl sulfate or a water-soluble carbodiimide after either pretreatment (assayed by primer extension; data not shown). Thus, in both forms of the t84D substrate, the major RNase E cleavage site is single-stranded. These observations rule out the possibility that this site is occluded by an alternative secondary structure. Second, the data in Fig. 3a, lane 6, show that boiling of t84D greatly increases the sensitivity of G residues 238* (the asterisk denotes a residue transcribed from the vector), 239*, 243*, 246*, 247*, 253, 256, 257, 259, 360, and 361 (see also Fig. 3b, lane 6). In contrast, these residues are virtually resistant to T1 in the renatured sample (Fig. 3a, lane 5). Residues 265 and 268, the latter being only weakly reactive, display slightly increased cleavage by T1 after boiling. The results with RNase CL3 are not quite as dramatic,

![Fig. 1. Model for the structure of the S20 mRNA (t79D) (8). Residues are numbered following the convention that residue 1 is the first residue transcribed from the natural P1 promoter. The model shown corresponds to the more abundant P2 mRNA which would initiate at residue 92. Lowercase letters denote residues transcribed from the vector. The two most prominent RNase E cleavage sites are shown by arrows. Oligonucleotide 481 is complementary to residues 265–279 extending from the bottom of stem III to the base of stem IV.](http://www.jbc.org/doi/10.1074/jbc.811076210)
but the reactivity of C residues 235\*, 244\*, 258, 261, and 295 increases significantly after boiling (Fig. 3a, lanes 7 and 8). The reactivity of residues 251–264 and 290–291 toward RNase T2 also increases after boiling (Fig. 3a, lanes 11 and 12). Attempts to obtain data on residues 226\*–233\* were complicated by two factors: first, the presence of significant levels of short RNAs, presumably abortive transcripts, which obscured the products of nuclease digestion; and second, the difficulty of resolving cDNAs differing by only a few residues from the full-length cDNA in primer extension assays (not shown). The first problem was largely eliminated by spin-column centrifugation (see "Experimental Procedures"). An experiment similar to that shown in Fig. 3a in which the products were resolved on a 16% gel showed that the reactivity of residues C231*, G234*, and C235* increased an average of 2.5-fold after boiling. In contrast, the reactivity of A230* toward RNase T2 was unaltered (data not shown).

Taken together, these data are consistent with a model for t84D in form A in which residues 233\*–248\* encoded by the vector form one arm of a stem (stem III*) paired with residues 253–267 (Fig. 4a). This stem is effectively grafted onto the remainder of the S20 mRNA at residue 268 and is distinguished by 2 G-U pairs and by three noncanonical A-G pairs in helix III*. Its \( \Delta G \) of formation, calculated as in Ref. 22, is only \(-4.4\) kcal/mol, a value which may underestimate the actual stability in view of the five nonstandard pairings whose contribution to stability is difficult to calculate. The presence of three A-G pairs seems surprising, but there are precedents in other RNAs (23–27). These pairs may account for the ease of transformation of form A into the more susceptible form B by boiling (see below).

Residues 271–276 are clearly single-stranded in both forms of t84D as C272\*, C274\*, and G276\* are highly sensitive toward RNases CL3 and T1, respectively. Likewise, residues 280–285 form a short stem (stem IV) which is part of the previously proposed model for the complete S20 mRNA (Fig. 1). The data obtained with V1 nuclease (Fig. 3a, lanes 9–10) generally support the model for stem III* well. Residues 235\*–265 in t84D (form A) and 15 60
t84D (form B) 24 96
A 2.9 2.4 2.4
B 15 7.9 7.8
C 140 130 86
194D A 2.3 ND <0.5
B 2.3 ND <0.5
C 131 ND 14
160D A 55 ND ND

\[\text{Effect of t84D on cleavage of 9S RNA}\]

Uniformly labeled 9S RNA (7) was incubated at a final concentration of 20 nM with 50 nM competitor, treated as described under “Experimental Procedures,” and digested with an AS-26 prepared from strain CF881 (0.1 mg/ml). Rates of formation of p5S were determined in duplicate (see "Experimental Procedures").

| Source of activity | \(\frac{\text{t87D}}{\text{t84D}}\) | \(\frac{\text{t160D}}{\text{t194D}}\) |
|-------------------|----------------|----------------|
| \(\text{I}\)      | 30            | 30            |
| \(\text{II}\)     | <0.5          | ND            |
| \(\text{III}\)    | 135           | ND            |

The rate is given as fmol per h of p5S RNA formed in a sample containing 125 ng of total protein.

TABLE I

| Source of activity | \(\frac{\text{t84D}}{\text{t194D}}\) | \(\frac{\text{t160D}}{\text{t194D}}\) |
|-------------------|----------------|----------------|
| \(\text{I}\)      | 2.3            | ND            |
| \(\text{II}\)     | ND             | <0.5          |
| \(\text{III}\)    | 131            | ND            |

\(^a\) Sources of activity are: I, AS-26 fraction from strain CF881; II, AS-26 fraction from strain GM402; III, purified Rnase protein. Details are given under “Experimental Procedures.”

\(^b\) The initial rate of cleavage of boiled t87D is greatly reduced but recovers during the assay (4).

\(^c\) ND, not determined.

\(^d\) Uniformly labeled 9S RNA (7) was incubated at a final concentration of 20 nM with 50 nM competitor, treated as described under “Experimental Procedures,” and digested with an AS-26 prepared from strain CF881 (0.1 mg/ml). Rates of formation of p5S were determined in duplicate (see "Experimental Procedures").

\(\text{Effect of t84D on cleavage of 9S RNA}\)

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cooling. The increased reactivity of G360 and G361 after boiling (Fig. 3, compare lanes 5 and 6) may be due to disruption of an interaction across the loop of stem VIa to C367 and C368 as shown in Fig. 4. This putative interaction does not, in any event, correlate with sensitivity or resistance to RNase E cleavage at residues 300/301/302 (cf. t194D below and t94D in Ref. 10). Thus in form B, t84D would resemble the model shown in Fig. 4 except that residues 226–268 would be essentially single-stranded. The data for both forms of t84D support the model in Fig. 4 except for C342 which is cleaved by RNase CL3 despite its predicted location in stem VI (Fig. 3b, lanes 7 and 8). This residue’s relative reactivity toward dimethyl sulfate was not as pronounced (data not shown). It is possible that the A-rich environment of C342 accounts for its high sensitivity to CL3.

Structure Mapping of t194D and t175D—Inspection of the sequence of t194D showed that a direct repeat in the S20 mRNA, ACAAGCU, at residues 260–267 and 341–348 is complementary to residues 412–419. In principle, residues 260–267 could pair with residues 412–419 in the lower arm of stem VI, displacing part of stem VI and sequestering the 5′ end of t194D. Alternatively, some of residues 260–267 could form a base triple interaction with stem VI. Either eventuality would almost completely sequester the 5′ end of t194D and would constrain residues 271–279 within a closed loop. Either of these is clearly distinguishable from a simple model in which t194D is single-stranded between residues 258 and 279 (Fig. 4b) but otherwise resembles t87D in Fig. 1. Moreover, t175D lacks residues 260–267 and should not be able to form such alternative structures.

RNA substrates t194D and t175D were 5′-end-labeled (see “Experimental Procedures”) and subjected to limited ribonuclease-
FIG. 4. Models for the secondary structures of t84D (form A) and t194D. Residues are numbered taking the 3' end as residue 447 in order to maintain parallels with the full-length S20 substrate in Fig. 1. Panel a shows t84D in form A (renatured) and panel b shows t194D. Lowercase letters (residues 226–247 in t84D and residue 258 in t194D) denote residues contributed by the vector which are not part of the natural S20 mRNA. The arrows indicate the sites of RNase E cleavage. The dotted lines in the loop of stem VIa in t84D indicate a potential base pairing interaction (see the text).
formation of stem VI (cf. pGM97 in Ref. 10) in the latter but would be incompatible with alternative models for t194D discussed above. These derivative substrates were cleaved at rates essentially identical to those measured for t194D itself (data not shown). Moreover, structure mapping experiments (not shown) demonstrated the disruption of stem VI in t194/94D and its retention in t194/97D.

Although t175D was expected to be initiated at G\(^{266}\), many transcripts appeared to be one residue shorter than expected as if initiation were also occurring at residue 269. As a consequence, the mapping data for t175D are not as clean as for the other RNAs and were particularly complex near its 5′ end. Nonetheless, they show that the sensitivity of t175D to nucleo-

digase digestion is similar to that for t194D, notably at residue C\(^{300}\) but with some exceptions at the bases of stems IV and V (Fig. 5b).

**DISCUSSION**

A substantial body of evidence has accumulated to show that several features in an RNA substrate control the efficiency of RNase E action at a given site. The single-stranded character and nucleotide composition, particularly richness in A and U, at the cleavage site are clearly very important determinants of how efficiently a site is recognized (5–10). Likewise, secondary structures can influence the recognition of a cleavage site by at least two means. Steric hindrance imposed by adjacent stem-loops could easily impede the binding of RNase E to its target site in view of the large size of this enzyme, particularly in its complexed form (“the degradosome”; cf. Refs. 28–30). The observation that relatively short oligoribonucleotides containing the RNase E site in RNA1 are much more efficiently cleaved than RNA1 itself has been interpreted to mean that secondary structures usually found near RNase E cleavage sites are intrinsically inhibitory. Secondary structures can also inhibit RNase E action by the simple means of occluding cleavage sites. The exposure of an otherwise cryptic cleavage site at residues 340/341 in the S20 mRNA by mutational destabilization of stem VI has provided evidence for this effect of secondary structure (10).

The results presented here suggest that the recognition and cleavage of substrates by RNase E is more complex than previously thought. The data show that the ability of RNase E to cleave a given site is also very strongly dependent on a third factor besides the nature of the cleavage site itself and its adjacent secondary structures, namely the “upstream” 5′ end. The substrates t84D, t194D, and t175D display reduced affinity for RNase E at the 300/301/302 site used *in vivo* and *in vitro* (8), although the single-stranded character of the cleavage site is retained. Moreover, most of the analogous secondary structural motifs present in the full-length S20 mRNA are also encompassed in these substrates, including stem-loop IV 5′ to the cleavage site and stem loops V, VI, VIa, Vlb, and VII 3′ to the cleavage site. Rather, only the termini 5′ to position 280 differ in these low affinity substrates relative to the full-length S20 mRNA. Removal of the “inhibitory” termini, either by RNase H treatment (t84D or t194D), by deletion (t160), or by boiling (t84D), enhances their rates of cleavage markedly. In contrast, t160D whose 5′ end is only two residues from a minor natural site for RNase E is cut efficiently. This latter finding and the reversible “silencing” of t84D show that there is no correlation between the presence of a proximal terminal triphosphate and efficiency of cleavage, unlike the situation in a derivative of RNA1 (31). The activation provided by truncation of substrates with RNase H and oligonucleotide 481 probably destabilizes stem IV, a structure of marginal stability ($\Delta G = -2.7$ kcal/mol) (8). This would improve access to two minor RNase E sites in stem IV and would remove residues
Our observations can be explained if RNase E contacts substrates at one or more sites in addition to the cleavage site at residues 300/301/302. They suggest a model in which RNase E recognizes “internal” sites in a substrate (i.e., sites which are >20 residues from the 5’ end or are separated from the 5’ end by one or more intervening stem loops) in two stages. The first stage would be an initial, perhaps rate-limiting, recognition phase requiring exposure of an unstructured 5’ end on the substrate, ideally containing an RNase E site (see below). The initial binding/cleavage event would engage RNase E on the substrate. Substrates such as t84D, t194D, and t175D are apparently poorly recognized in this step but for different reasons. The 5’ end of t84D (form A) folds to present a secondary structure somewhat reminiscent of the 5’-terminal stem-loop of the ompA mRNA which is resistant to rne-dependent decay in vivo (11–14). In both t194D and t175D the 5’ ends are single-stranded, but no RNase E cleavage sites have been mapped to the region between residues 250 and 282 either in the S20 mRNA itself (4) or in either of these substrates (data not shown). Thus the initial recognition phase requires not only a single-stranded target but one which contains a cleavage site as well. The second phase would be the recognition of the more distal RNase E cleavage site while the enzyme remains bound to the product of the first cleavage. The presence of a strong RNA binding site in the Rne protein (20, 32, 33) could facilitate the retention of products (34). Structural and steric factors such as the positioning of adjacent secondary and tertiary structural elements (10) as well as subtle features of the nucleotide sequence at the cleavage site (5) would govern the efficiency of the second step. In mRNA substrates containing multiple RNase E sites, cleavage at one site would in this model constitute the initial recognition phase for cleavage at the next site. In effect, there would be a form of internal cooperativity in an mRNA such that prior cleavage at a more 5’ site would permit efficient recognition of a distal site. Each endonucleolytic cleavage could be coupled to subsequent 3’ to 5’ degradation initiated at the newly exposed 3’-terminus generated by RNase E (28–30). This process could drive the release of products from RNase E to permit the next cycle of endonucleolytic cleavage. This model implicitly predicts that RNase E contains at least two subunits of Rne. Although there are no data bearing directly on this point, the size of the “degradosome” and the apparent stoichiometry of Rne in this complex (28–30) do not exclude this possibility.

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