Studies on the Chemical Structure of Yeast Amino Acid Acceptor Ribonucleic Acids

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Elucidation of the nucleotide sequence of amino acid acceptor ribonucleic acid may reveal the structural basis of its amino acid specificity. The task appears feasible in the near future because of the low molecular weight attributed to S-RNA and because of the purity of the S-RNA preparations achieved so far.

Ingram and Pierce (1), McCully and Cantoni (2), and Bell, Tomlinson, and Toner (3) have determined the base ratios and oligonucleotide distribution in specific enzymatic digests of crude S-RNA preparations, and Holley et al. (4) reported the base ratios and chromatographic profiles of enzymatic digests of purified alanine-, valine-, and tyrosine-S-RNA. Lagerkvist and Berg investigated the nucleotide sequences adjacent to the pCpCpA end of crude S-RNA (5), and of leucine- and isoleucine-S-RNA (6).

The studies on purified S-RNA revealed significant differences between the nucleotide sequences of individual amino acid acceptor RNA preparations (Holley et al. (4); Berg, Lagerkvist, and Dieckmann (6)).

This report describes the quantitative determination of the major mono- and oligonucleotides in pancreatic ribonuclease digests (7) from yeast alanine- and tyrosine-S-RNA purified by countercurrent distribution with two solvent systems (8, 9).

Our results indicate large variations between the nucleotide sequences of these two S-RNA preparations, in agreement with the data obtained by other methods for the same RNAs by Holley (7).

EXPERIMENTAL PROCEDURE

S-RNA—This material was obtained from yeast according to the procedure of Holley et al. (10). The final preparation was re-extracted with phenol in order to eliminate possible traces of nuclease activity (9).

Aminoacyl S-RNA Synthetases—The pH 5 fraction synthetases were prepared from rat liver homogenates as described (8, 11).

*A preliminary report of this investigation was presented at the meeting of the Federation of American Societies for Experimental Biology, April 16 to 20, 1963, Atlantic City, New Jersey (Abstracts, 410).

1 The abbreviation used is: S-RNA, soluble or transfer ribonucleic acid.

200 - Transfer Countercurrent Distribution of S-RNAs—A modification of Kirby’s solvent system was used (8). The countercurrent apparatus (model 520, E. C. Apparatus Company, Swarthmore, Pennsylvania) was charged with 10 ml of lower phase (200 tubes) and 10 ml of upper phase (tubes 1 to 25). About 1 g of S-RNA was dissolved in 60 ml of each upper and lower phase, and introduced in tubes 1 to 6 in place of the solvent. A small portion of undissolved sample was removed and re-extracted with 20 ml of each phase and placed into tubes 7 and 8. After 200 transfers (at 23°C) with a 3-minute equilibration and 4-minute separation time, 40 fractions were obtained by pooling five consecutive tubes along the extraction train. The fractions were then extracted individually in a 500-ml separatory funnel, with 100 ml of ether, and equal amounts of the lower phase were placed in three 50-ml polypropylene centrifuge tubes. After the addition of 13 ml of 2-methoxyethanol to each tube, stirring with a glass rod, and centrifugation for 5 minutes at 10,000 r.p.m. in a Servall SS-1 centrifuge, the supernatant fluid was extracted with 125 ml of 1-butanol and 150 ml of ether. The aqueous phase was then dialyzed and tested for amino acid acceptor activity and for absorbance at 260 μm. After dialysis, the RNA fractions with maximal specific activity for incorporation of alanine and tyrosine were isolated as described (9) and redistributed in another solvent system.

 Redistribution of Alanine- and Tyrosine-S-RNA—The solvent system of Apgar et al. (9) was modified to contain 45 ml of isopropyl alcohol per 100 ml of 1.85 M phosphate buffer, pH 6.9. The most active amino acid acceptor S-RNA preparations (see above) were placed in tube 1 (or 1 and 2) of the E. C. model 520 countercurrent apparatus. The most active amino acid acceptor S-RNA preparations (see above) were placed in tube 1 (or 1 and 2) of the E. C. model 520

3 The amino acid incorporation assays were performed according to Holley et al. (10) with the following changes. The incubation mixture consisted of 100 amoles of Tris-HCl, pH 7.5; 10 amoles of potassium EDTA; 0.05 ac of 14C-amino acid; 10 amoles of MgCl2; 10 μmoles of KCl; 2.5 μmoles of sodium ATP, pH 6.8; S-RNA; enzyme; and water to a total volume of 1 ml. Incubation time was 20 minutes at 37°C. Radioactive amino acids were purchased from Nuclear-Chicago, Inc., and have the following specific activities: L-alanine (uniformly labeled), 4.24 mc per mmole; L-tyrosine (uniformly labeled), 22.0 mc per mmole. One millimicromolecule gave approximately 425 c.p.m. under the counting conditions (99% efficiency).
fractionator as described above. Because of the low partition coefficient (8, 9) of the S-RNA preparations, the upper phase was recycled several times in one run. Thus alanine-S-RNA was distributed for 500 transfers, and the fractions with the highest incorporating activity were pooled and redistributed in fresh solvent for 800 more transfers. Similarly, tyrosine-S-RNA was purified by a 650- and subsequent 660-transfer distribution. At the end of the runs, the dialyzed fractions were concentrated to a small volume in a flash evaporator at 37° and the RNA was precipitated with alcohol as described above.

**Enzymatic Digestion of S-RNA Preparations and Fractionation of Products**—About 8 to 10 mg of S-RNA and 0.3 mg of RNase (Worthington, twice recrystallized from alcohol) were dissolved in 1.15 ml of 0.1 M Tris-chloride, pH 7.5, and held at 24° for 6 hours. The concentration of RNA was determined by alkaline hydrolysis (n KOH for 22 hours at 24°) of aliquots of each digest and of the original S-RNA. A value for absorbance at 260 μm of 24.0 for a 0.1 % solution (neutral pH) was used; after alkaline hydrolysis, the A_{260} (in n KOH) of the original mixture and of tyrosine- and alanine-RNA increased by 30, 36, and 31%, respectively. All measurements were made in a Beckman DU spectrophotometer with 1-cm silica cells and appropriate blanks. Duplicate aliquote (0.5 ml) of each digest were fractionated by two-dimensional electrophoresis and paper chromatography (7). Contact prints of the maps were prepared according to the method of Smith and Allen (12).

The isolated compounds were identified by comparison with standard maps prepared with similar digests of RNA from tobacco mosaic virus (7). For quantitative analysis, the major compounds located under ultraviolet light were cut out and eluted by shaking for 3 hours at 23° with 7 ml of 0.01 M sodium
FIG. 4 (left). 650-Transfer countercurrent distribution of tyrosine acceptor RNA fraction from two duplicate 200-transfer distributions such as shown in Fig. 1. This redistribution was performed in a modification of the solvent system described by Apgar et al. (9) (see "Experimental Procedure"). ○—○, milligrams of RNA per fraction; □—□, tyrosine acceptor activity.

acetate, pH 4.5, containing 0.15 unit of ribonuclease T2 per ml. Under these conditions oligonucleotides are completely hydrolyzed to mononucleoside 3'-phosphates (13). After addition of 0.2 ml of m sodium phosphate, pH 7.0, the total A260 values (volume and absorbance) were thus obtained for each spot.

New components in the S-RNA digests were identified by their positions on the map as well as by their A260 : A280, A260 : A290, and A260 : A400 ratios at pH 2 and 7.

RESULTS

The results of a 200-transfer countercurrent distribution of yeast S-RNA with a solvent system modified after Kirby (8, 14) are shown in Fig. 1. The recovery of ultraviolet absorbance at 260 mu was about 75%, and the recovery of alanine and tyrosine acceptor activity was 90%.

The most active fractions were redistributed twice more (Figs. 2 to 5). The final fractionation of each RNA resulted in weight and activity patterns close to those expected from theory for homogeneous compounds (15). As Table I shows, the specific activities of alanine- and tyrosine-S-RNA were thus increased to 63 and 66% of theory, respectively. The material selected for structural studies was pooled from the tubes in that region of the distribution pattern (Table I; Figs. 3 and 5) where essentially complete homogeneity was found. These samples were also assayed with 14C-labeled protein hydrolysates from algae and unlabeled alanine and tyrosine. In each case, no acceptor activity toward 14C-amino acids was observed, an indication of the absence of contaminating S-RNAs in the pooled fractions (9).

FIG. 5 (right). 660-Transfer countercurrent distribution of tyrosine acceptor RNA fraction derived as shown in Fig. 4. ○—○ milligrams of RNA per fraction; □—□, tyrosine acceptor activity; Δ—Δ, theoretical curve calculated for the partition coefficient of 3.85.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Increase in activity over original</th>
<th>Approximate amount of amino acid bound per mg of RNA</th>
<th>Approximate purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube No.</td>
<td>Fig.</td>
<td>-fold</td>
<td>pmol/mg</td>
</tr>
<tr>
<td>Starting mixture</td>
<td>0.85</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>21-35</td>
<td>1</td>
<td>14.0</td>
</tr>
<tr>
<td>76-95</td>
<td>2</td>
<td>25</td>
<td>21.0</td>
</tr>
<tr>
<td>56-80</td>
<td>3</td>
<td>23</td>
<td>22.0</td>
</tr>
<tr>
<td>Starting mixture</td>
<td>0.91</td>
<td>10</td>
<td>9.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>161-180</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>481-515</td>
<td>4</td>
<td>23</td>
<td>12.0</td>
</tr>
<tr>
<td>511-550</td>
<td>5</td>
<td>23</td>
<td>22.0</td>
</tr>
</tbody>
</table>

The distribution of products isolated from pancreatic RNase digests (of the original mixture as well as) of the two purified S-RNA preparations is shown in Fig. 6. The completion of enzymatic digestion was ascertained by the virtual absence of 2',3'-cyclic phosphate terminal pyrimidine mononucleosides (16). For quantitative analysis, the material in the mono-, di-, tri-, and major tetranucleotide spots was eluted with dilute RNase T2 and simultaneously hydrolyzed to mononucleoside 3'-phosphates. The total A260 per compound in 3 mg of S-RNA so fractionated is given in Table II. New components (absent from digests of high molecular weight RNA) were located by their positions on the maps as well as by their absorption ratios.
TABLE II

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Original mixture</th>
<th>Tyrosine-RNA</th>
<th>Alanine-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp</td>
<td>9.15</td>
<td>12.1</td>
<td>9.66</td>
</tr>
<tr>
<td>Up</td>
<td>6.93</td>
<td>7.0</td>
<td>7.97</td>
</tr>
<tr>
<td>GpCp</td>
<td>4.36</td>
<td>2.3</td>
<td>2.64</td>
</tr>
<tr>
<td>ApCp</td>
<td>3.04</td>
<td>1.3</td>
<td>0.32</td>
</tr>
<tr>
<td>ApUp</td>
<td>3.03</td>
<td>1.2</td>
<td>0.53</td>
</tr>
<tr>
<td>GpUp</td>
<td>5.02</td>
<td>2.3</td>
<td>4.28</td>
</tr>
<tr>
<td>ApApCp</td>
<td>1.18</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>(ApGp)Cp</td>
<td>2.34</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>ApApUp</td>
<td>2.82</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>(ApGp)Up</td>
<td>5.64</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>GpGpUp</td>
<td>4.51</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>GpGpCp</td>
<td>2.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ApGpGp)Cp</td>
<td>2.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ApGpGp)Up</td>
<td>2.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ApGpGp)Up</td>
<td>2.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Ribosyl-Up</td>
<td>1.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Identified by comparison with similarly digested RNA from tobacco mosaic virus; unidentified compounds are listed by numbers (Fig. 6).

† In terms of A260 at pH 7.0 (after hydrolysis to mononucleotides, see text) per 3 mg of S-RNA. This value (3 mg) corresponds to the average amount of S-RNA applied to one map. The reproducibility of these determinations is better than ±5% with the major mono-, di-, and trinucleotide spots, and better than ±8% with the other components. A dash (―) indicates that the compound was either absent or present in too low a concentration to be seen or measured or both (less than A260 of about 0.025 per ml).

‡ The alanine and tyrosine acceptor RNA preparations were about 63 and 66% pure, respectively. It is assumed (a) that the absorbances (24.0 for a 0.1% solution at neutral pH) and molecular weights (30,000) are the same for each S-RNA, and (b) that each active molecule is labeled during the assay. For the purpose of comparison, it is furthermore assumed that the A260 in a spot is entirely due to the major component present. The values reported by Holley et al. (4) are given (in parentheses) for alanine-S-RNA.

Fig. 6. Contact prints showing the fractionation on Whatman No. 3 paper of 3 mg of different S-RNA preparations. First dimension, electrophoresis (left to right) in 0.02 M ammonium formate, pH 2.7, for 17 hours at room temperature and 5 volts per cm; second dimension, paper chromatography, descending, with t-butyl alcohol-0.02 M ammonium formate (35:45, volume for volume), pH 3.7. The papers were serrated so that the solvent could run off the paper without edge effects. Running time was 28 to 36 hours at 23°C. Unidentified spots (the amounts of which were not determined) are indicated by dashed lines.
solvent systems for the distributions yielded preparations the purities of which are comparable to those obtained by others (9). Furthermore, the use of different solvent systems reduces the total number of transfers required for the purification.

The estimation of purity of amino acid acceptor RNAs usually involves the assumptions (9) (a) that the absorbances and molecular weights (30,000) of the different RNA preparations are the same as those of the original mixture, and (b) that each active RNA molecule is labeled during assay. Our estimations of the purity of our preparations thus do not use the same criteria employed by others, who use the absence of contaminating amino acid acceptor activity as a criterion (17) rather than the ratio of moles of specific amino acid incorporated per mole of S-RNA. If the absence of other amino acid acceptor RNA activity is used as a measure of purity (17), then our preparations should be considered 100% pure. The method employed here for the fractionation of RNase digests of S-RNA yields about 16 clearly resolved spots which were identified by comparison with standard maps from similar digests prepared with high molecular weight RNA (Fig. 6). This identification does not rule out contamination by small amounts of unidentified compounds with similar optical, chromatographic, and electrophoretic properties. The small amounts of the S-RNA fractions available do not permit thorough tests of the purity of the spots isolated from the maps. In spite of this limitation and the 65% purity of our S-RNA, it is quite evident that there must be striking differences between the nucleotide sequences of alanine- and tyrosine-S-RNA.

Other mapping procedures have been employed by Ingram and Pierce (1) for the fractionation of RNase digests of unfraccionated yeast S-RNA, but the resolution of the oligonucleotide spots was not very clear (Fig. 1 of (1)). The authors stated that this may have been due to insufficient resolution of the major compounds or “measuring due to the presence of additional components” in those areas on the maps. As Fig. 6 shows for our maps, the GpUp spot in unfractionated and tyrosine-S-RNA clearly contains several components; the same holds true for GpUp in tyrosine-S-RNA and (ApGp)Up in alanine-S-RNA. However, these spots on our maps are sufficiently isolated to permit their subsequent elution and determination of the additional components by additional procedures.

To alleviate “smearing,” Ingram and Pierce recommended (1) the removal of terminal phosphate groups in the RNase digests before mapping; but a later communication by another author from the same laboratory indicates that dephosphorylation is not a prerequisite for good resolution (18).

McCully and Cantoni (2) also dephosphorylated RNase digests of liver S-RNA because this facilitated their fractionation by column chromatography on Dowex 1 with a formate gradient. However, between lyophilizations required to remove formic acid, the residues were dissolved in N HCl. In view of various reports of the sensitivity of minor bases to acid hydrolysis (19, 20), it would seem advisable to avoid N HCl or concentrated formic acid solutions where minor bases are involved, especially if such determinations require large amounts of S-RNA (2). Since the stability to alkali of minor bases is not well known, it seems preferable to use enzymatic hydrolysis for the determination of base ratios. Ribonuclease T2 (21, 22) is quite satisfactory in this respect.3

3 G. W. Rushizky and H. A. Sober, unpublished observations.

Column chromatography on DEAE-cellulose with ammonium carbonate buffers (23) has been successfully used by Lagerkvist and Berg (5) and Berg et al. (6) to resolve doubly labeled oligonucleotides occurring next to the common terminal ...,pCpCpA sequence of S-RNA. Considerable heterogeneity in the nucleotide sequences at the amino acid acceptor end of unfraccionated S-RNA was found; leucine- and isoleucine-S-RNA have different sequences in that part of the chain. However, this approach is limited to sequences at the amino acid acceptor terminus and is not applicable to the determination of nucleotide sequences in the rest of the chain.

Holley et al. (4) and Bell et al. (3) have separated RNase digests of S-RNA on DEAE-Sephadex and DEAE-cellulose in 7 M urea, respectively. Although both methods yield good fractions, most of the peaks obtained appear to contain mixtures of compounds, necessitating further separations. By comparison, the approach used in this study is simpler but limited to oligonucleotides no larger than tetramers. A comparison of our results (Table II) with those reported recently by Holley et al. (4) shows that both procedures yield similar values. Thus, there are more GpUp and fewer ApUp sequences in alanine- than in unfraccionated S-RNA. Neither tyrosine- nor alanine-S-RNA preparations contain ApApCp, whereas there is 0.3 mole of this compound per mole of unfraccionated S-RNA. It is of interest that the most pronounced differences between the purified and unfractionated S-RNAs are found among compounds containing minor bases (Fig. 6; Table II).

SUMMARY

The purification of alanine and tyrosine acceptor ribonucleic acid by countercurrent distribution in two different systems is described. After hydrolysis by pancreatic ribonuclease, digests of both RNA preparations were fractionated by a two-dimensional mapping procedure. Significant differences between the nucleotide sequences of alanine and tyrosine acceptor RNA were thus found.

REFERENCES

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