SYNTHESIS AND CODING PROPERTIES OF POLY(c^1A), POLY(c^3A), POLY(c^7A) AND POLY(h^6A)

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Summary: The homopolynucleotides poly(c^1A), poly(c^3A), poly(c^7A) and poly(h^6A)[++] were synthesized from their corresponding nucleoside diphosphates using polynucleotide phosphorylase. With the exception of poly(h^6A), which displayed no hypochromicity, the homopolynucleotides showed melting profiles similar to poly(A). All these polynucleotides, poly(h^6A), poly(c^7A), poly(c^3A) and poly(c^1A) stimulated the binding of Lys-tRNA to ribosomes; the coding activity of poly(c^1A), however, was very low. Poly(h^6A) was found to be less specific for Lys-tRNA than poly(A). The data supports the exclusive formation of Watson-Crick type base pairs and contradicts Hoogsteen base pairing in codon-anticodon recognition. Since, however, poly(h^6A), which can form only one hydrogen bridge per base pair, stimulated the binding of Lys-tRNA comparably to poly(A), the coding activity of the homopolynucleotides tested is discussed in respect to their secondary structure as well as to the pK-values of their 6-amino groups.

Introduction: According to the Watson-Crick hypothesis, a complex between the mRNA codon and the tRNA anticodon should be stabilized by the formation of 6 - 9 hydrogen bridges between complementary bases. Whereas the position of the hydrogen bridges in a G:C base pair is fixed, there is evidence that in an A:U base pair complex formation according to Hoogsteen (1) may be an alternative possibility to the Watson-Crick type complex. In order to examine whether Hoogsteen base pairs can be formed during codon-anticodon recognition, we synthesized homopolynucleotides containing the nucleosides shown in Fig. 1.

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++ These compounds are symbolized, according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (Europ. J. Biochem. 15 (1970) 203) as follows: c^1Ado = 1-deazaadenosine, c^3Ado = 3-deazaadenosine, c^7Ado = 7-deazaadenosine = tubercidine, h^6Ado = purineriboside = nebularin (see Fig. 1).

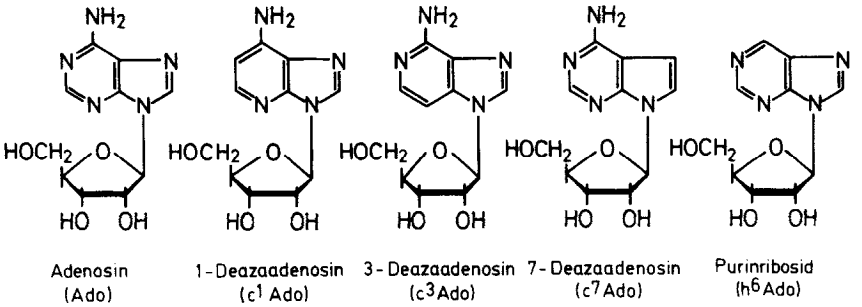

Fig. 1. Adenosine and its analogues studied.

Provided the formation of a stable base pair requires at least two hydrogen-bridges, the complex c^7Ado:Urd (2) could be only a Watson-Crick type base pair, whereas the complex c^1Ado:Urd could be stabilized only in a Hoogsteen base pair. The pK-values of the 6-amino groups have to be considered: 3.45 (Ado), 3.65 (c^7Ado), 3.80 (c^3Ado) and 4.75 (c^1Ado) (3). Poly(h^6A) was synthesized to examine whether a codon-anticodon complex might be formed, if only one hydrogen bridge per base pair is possible. Synthesis, melting profiles and some coding properties of these homopolynucleotides are briefly reported.

Materials and Methods: Purineriboside and tubercidine were obtained from commercial sources, 1-deazaadenosine and 3-deazaadenosine were synthesized according to modifications (3) of the procedures of Roos and Salemink (4) and Rousseau and Robins (5). [^{3}H]Lysine(spec. act. = 3 Ci/nmole) was obtained from the Radiochemical Centre, Amersham.

Phosphotransferase from carrots was prepared according to the method of Brunngraber and Chargaff (6), and Micrococcus luteus polynucleotide phosphorylase was isolated as described (7). E. coli B ribosomes and [^{3}H]AA-tRNA s were prepared by known procedures (8).

Synthesis of 5'-nucleotides: Mixtures composed as follows were incubated for 6 h at 37 °: 0.1 ml Triton X-100 (250 mg/l), 0.5 ml 2 M CH_3COONa pH 5.0, 1.0 ml 2 M phenylphosphate, 0.2 ml 0.5 M nucleoside and 1.0 ml phosphotransferase (50 U/ml). After incubation, the mixture was heated to 80 ° for 3 min and streaked on 4 Schleicher and Schüll No. 2316 paper sheets (50 cm x 60 cm). Chromatograms were developed in butanol: acetic acid: H_2O=5:2:3. The slowest moving band

Table 1. Yields and data of the nucleoside-5'-phosphates synthesized with phosphotransferase. Paper electrophoresis was performed on Schleicher and Schüll paper No. 2316 on a flat plate apperatus in 0.02 M $HCOONH_4$ pH 2.5 or 0.1 M $(C_2H_5)_3$ NH·HCO_3 pH 7.5, resp. R_E of Up = 1.00.

Nucleotide	Yield %	$R_E^{pH\,2.5}$	$R_E^{pH\,7.5}$	λ_{max}	λ_{min}	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$
pc^1A	48	0	0.75	277, 262	269, 237	0.68	0.76	0.38
pc^3A	39	0	0.73	261, 267(sh)	236	0.76	0.75	0.44
pc^7A	61	0	0.73	269	243	0.57	1.00	0.59
ph^6A	75	0.57	0.73	263	235	0.80	0.17	0.07

Table 2. Yields and data of the nucleoside diphosphates

Nucleoside diphosphate	Yield %	$R_E^{pH\,2.5}$	$R_E^{pH\,7.5}$	λ_{max}	λ_{min}	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$
ppc^1A	19	0.87	0.82	276, 262	270, 236	0.70	0.86	0.52
ppc^3A	21	0.82	0.80	261	231	0.74	0.90	0.43
ppc^7A	68	0.92	1.10	269	241	0.58	0.96	0.47
pph^6A	58	1.17	1.16	262	221	0.69	0.12	0.05

Table 3. Yields and data of the homopolynucleotide

Polynucleotide	Yield %	λ_{max}	λ_{min}	Average chain length	T_m^o	Hypochromicity %
poly(A)	30	255	229	80 - 100	27	26
poly(c^1A)	12	278, 262	271, 245	70 - 90	65	236
poly(c^3A)	10	260	243	70 - 90	69	36
poly(c^7A)	23	269	246	80 - 100	60	118
poly(h^6A)	27	262	223	70 - 100	-	0

contained the nucleoside-5'-phosphate. Recoveries and spectral data of nucleotides synthesized are listed in Table 1.

Synthesis of nucleoside diphosphates: The procedure of Hoard and Ott (9) was closely followed. Recoveries and analytical data of these compounds are seen in Table 2.

Synthesis of homopolynucleotides: Incubation mixtures contained in 1.0 ml : 200 µl 20 mM nucleoside diphosphate-K, 20 µl 10 mM EDTA-K, 5 µl 1 M $MgCl_2$, 500 µl 1 M Tris-HCl pH 9.3, and 2.5 units of PNPase. The mixtures were incubated for 3 h at 37 °, heated to 80 ° for 3 min, extracted 3 times with phenol and 3 times with ether. Polynucleotide was separated from the monomer by chromatography on Sephadex G-50. Yields and data are listed in Table 3. Chain length and purity of homopolynucleotides were determined as described (10). The UV-spectra are shown in Fig. 2.

Melting curves were measured in 0.1 M NaCl, 10 mM $MgCl_2$, 50 mM cacodylate-Na pH 7.0 in a Beckman DB G spectrophotometer. The temperature was controlled inside the cell with a thermocouple. Hypochromicities were calculated from the melting curves.

Polynucleotide-dependent binding of AA-tRNA to ribosomes was assayed as described (8). 100 µl reaction mixtures contained: 2.4 A_{260} units ribosomes (washed 5 times with 0.5 M NH_4Cl), 0.71 A_{260} units = 1.42 nmoles tRNA 0.7 % charged with [^{3}H]Lysine (spec. act. = 3 Ci/nmole), 10 mM Tris-HCl pH 7.8, 60 mM KCl, 10 mM $MgCl_2$ and varying amounts of polynucleotide. Reaction mixtures were incubated at 37 ° for 20 min. The binding curves are shown in Fig. 3.

Results and Discussion: For the preparation of the nucleoside phosphates enzymatic phosphorylation was preferred to known chemical procedures (11), since the latter ones resulted in phosphorylation of the base moiety of c^1Ado, c^3Ado and h^6Ado. For the synthesis of the nucleoside diphosphates, however, chemical phosphorylation was used, since neither nucleoside monophosphate kinase (12) nor myokinase (13) gave satisfactory yields of the Ado-analogue diphosphates. The PNPase-catalyzed polymerisation was dependent on the use of potassium salts of the substrate and higher Tris-HCl concentrations compared to poly(A) synthesis.

The T_m-values of poly(c^7A), poly(c^1A) and poly(c^3A) are higher than the T_m of poly(A) (Table 3), pointing to a more stable secondary structure of these polynucleotides. Poly(h^6A), however, having no exocyclic group on the base portion showed no hypochromicity between 5 and 95 °.

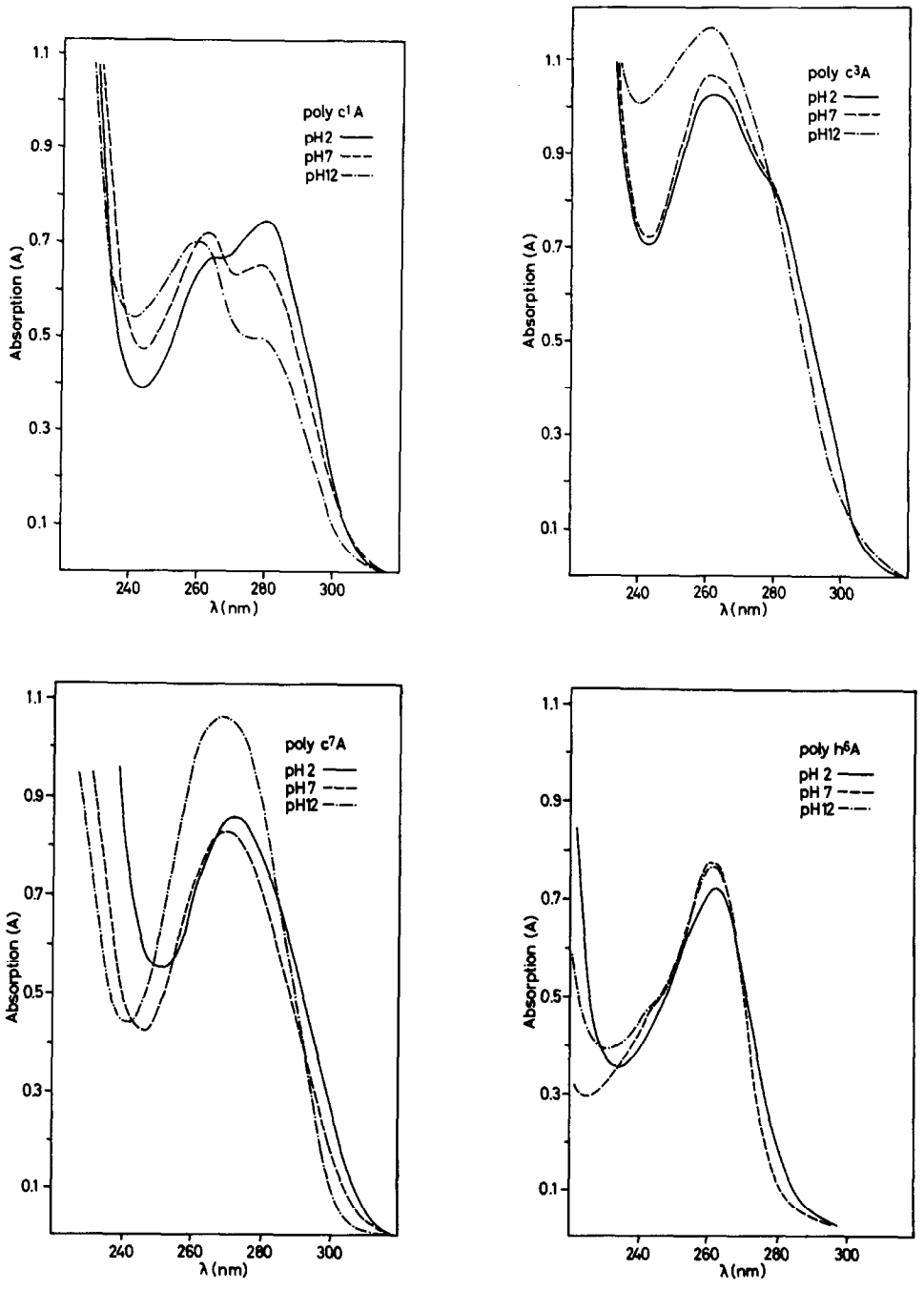

Fig. 2. UV-spectra of the homopolynucleotides at different pH-values.

It is concluded, therefore, that poly(h^6A) comparably to poly(c^7purine) has very little secondary structure. Thus the exocyclic group may play an important role for the stacking properties of polynucleotides as postu-

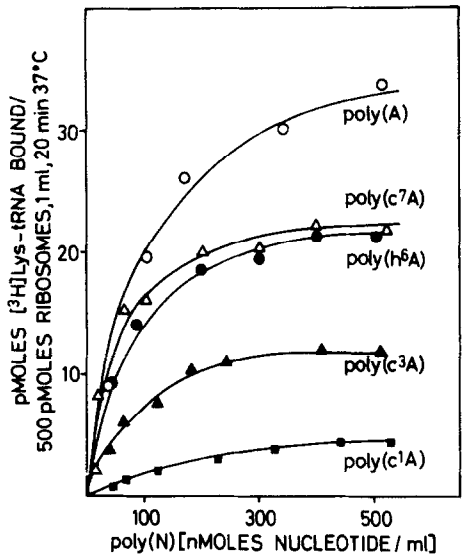

Fig. 3. Stimulation of Lys-tRNA binding to ribosomes by homopolynucleotides containing analogues of Ado. The minus polynucleotide blanks, i.e. 3-5 pmoles Lys-tRNA, have been substracted.

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lated by Bugg et al. (15) and shown for polynucleotides containing 2,6-bis-methylthiopurine nucleosides by Ikehara et al. (16).

Poly(c^7A) stimulated coded binding of Lys-tRNA to ribosomes to a similar extent as poly(A). Its somewhat lower coding activity might be due to a decreased binding to ribosomes. It is known (17) that poly(A) is bound to ribosomes only in the presence of $tRNA^{Lys}$, may be because the secondary structure of the polynucleotide has to be arranged into a conformation suitable for ribosomal binding. This rearrangement should depend on the stability of the secondary structure of the polynucleotide. Poly(c^1A) on the other hand, which could form two hydrogen bridges only in the way proposed by Hoogsteen, displays very low coding activity. The data support Watson-Crick type base pairing in codon-anticodon recognition. With both types of pairing, the stability of the complexes depends in part on the acidity of the 6-amino group, the pK-value of which is shifted from 3.45 (Ado) to 4.75 (c^1Ado). Poly(c^3A), which again can form Watson-Crick type hydrogen-bridges but shows an intermediate pK of 3.80 for the amino group, displays about 30 % of the coding activity compared to poly(A). Therefore, the very low stimulation of Lys-tRNA binding by poly(c^1A)

Table 4. Specificity of poly(h^6A) and poly(c^7A) tested in AA-tRNA binding. Triplicate binding assays as described in the text contained saturating amounts of polynucleotides and tRNA, charged with 19 nonlabelled plus one amino acid charged and labelled as follows: 0.47% [^{3}H] Lys spec. act. = 2815 Ci/mole, 1.89% [^{3}H] Arg spec. act. = 1612 Ci/mole, 1.22% [^{14}C] Glu spec. act. = 209 Ci/mole, 0.65% [^{14}C]AsN spec. act. = 218 Ci/mole, 2.73% [^{14}C]Gly spec. act. = 53 Ci/mole, 1.36% [^{14}C]Pro spec. act. = 265 Ci/mole, 1.52% [^{14}C]Phe spec. act. = 513 Ci/mole. Counting efficiency was 13.1% for ^{3}H and 51.5% for ^{14}C.

AA-tRNA	Lys-	Arg-	Glu-	Asn-	Gly-	Pro-	Phe-
Codons	AA(A,G)	AG(A,G)(CG(U,C,A,G))	GA(A,G)	AA(U,C)	GG(U,C,A,G)	CC(U,C,A,G)	UU(U,C)
Polynucleotide	pmoles AA-tRNA bound/.5 nmoles ribosomes, ml, 20'37°						
None	5.6	27.4	2.1	3.5	19.0	6.2	10.2
poly(A)	29.2	24.8	2.2	3.4	16.4	5.5	7.8
poly(h^6A)	27.0	46.0	1.8	3.0	11.6	5.1	11.4
poly(c^7A)	10.4	27.6	1.6	3.2	-	2.4	10.2
poly(C)	-	-	-	-	-	25.4	-
poly(U)	-	-	-	-	-	-	202.2
poly(A, U, G)	-	-	8.3	7.5	43.8	-	-

compared to both poly(A) and poly(h^6A) might be attributed to the formation of only one hydrogen bridge and, moreover, to the low acidity of the 6-amino group.

To our surprise poly(h^6A), which can form only one hydrogen bridge per base pair showed efficient codon activity (Fig. 3). To check whether this activity was specific for Lys-tRNA, we examined the binding of other AA-tRNAs as listed in Table 4. The purine riboside can substitute best for adenosine. In the case of Arg-tRNA, however, it acts very well, most likely in place of a G in the middle position of the codons AGA and AGG, whereas it does not replace G in the first position of the Glu-codons GAA and GAG. To a very low extent, h^6As reproducibly seem to substitute even for 2 or 3 Us in case of the Phe-codons UUU and UUC. h^6A

might thus form hydrogen bridges with U (Lys-), C (Arg-) or much less with A (Phe-tRNA). The h^6A:U pair appears to be the most stable one, whereas no pairing seems to occur with G (Pro-tRNA). The coding potential of h^6A appears similar so far to that of 7-deazapurineriboside, which in polynucleotides was found to substitute for A and G (18). In the assays reported, c^7A shows the specificity of A.

The coding activity of purine-homopolynucleotides forming only one hydrogen bridge per base pair seems to be decreased by secondary structure. The pyrimidine-polynucleotide poly(c^3U) was found to be inactive in the binding of Phe-tRNA (19), whereas poly(h^4U) was active. In the course of binding to the ribosome, poly(h^6A) may go from a random coil structure to a stacked conformation. The stacking energy gained might be sufficient to compensate for the formation of only three hydrogen bridges in the codon-anticodon complex. Four nucleobases are by now known to show coding activity although they cannot form more than three hydrogen bridges per coding triplet: h^6Pu (18), h^4U (20), h^6A, and to a low extent c^1A. We are trying to check the ideas of direct base pairing and role(s) of ribosomal protein(s) in codon-anticodon recognition.

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References.

1. K. Hoogsteen, Acta Cryst. 16 (1963) 907.
2. M. Ikehara and F. Fukui, J. Mol. Biol. 38 (1968) 437.
3. L. Hagenberg, Diplomarbeit Göttingen 1972.
4. K.B. de Roos and C.A. Salemink, Recueil 88 (1969) 1263.
5. R.J. Rousseau and R.K. Robins, Heterocyclic Chem. 21 (1965) 196.
6. E.F. Brunngraber and E. Chargaff, J. Biol. Chem. 242 (1967) 4834.
7. H. Schetters, H.G. Gassen and H. Matthaei, Biochem. Biophys. Acta 272 (1972) 549.
8. F. Gros and H. Matthaei in "Practical Molecular Genetics", Springer Verlag in press.
9. D.E. Hoard and D.G. Ott, J. Am. Chem. Soc. 87 (1965) 1785.
10. H.G. Gassen and W. Leifer, Z. Anal. Chem. 252 (1970) 337.
11. M. Yoshikawa, T. Kato and T. Takeniski, Tetrahedron Letters 50 (1967) 5065.
12. S.L. Strominger, L.A. Heppel and E.S. Maxwell Biochem. Biophys. Acta 32 (1959) 412.
13. J. Kohlschein and H.G. Gassen, Manuscript in preparation.

14. D. C. Ward and E. Reich, J. Biol. Chem. 247 (1972) 705.
15. C. E. Bugg, J. M. Thomas, M. Sundaralingam and S. T. Rao, Biopolymers 10 (1971) 175.
16. M. Ikehara and M. Hattori, Biochem. Biophys. Acta 281 (1972) 11.
17. D. Hatfield, Cold Spring Harbor Symp. 31 (1966) 619.
18. D. Grünberger, D. C. Ward and E. Reich, J. Biol. Chem. 247 (1972) 720.
19. H. G. Gassen and H. Schetters, Manuscript in preparation.
20. H. G. Gassen, H. Schetters and H. Matthaei, Biochem. Biophys. Acta 272 (1972) 560.