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Complex formation between ribosomal protein S1, oligo- and polynucleotides: chain length dependence and base specificity

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ABSTRACT

In order to examine the nature of the complex formation between the ribosomal protein S1 and nucleic acids three methods were used: Inhibition of the reaction of N-ethyl|2.3^{14}C|-maleimide with S1 by the addition of oligonucleotides; adsorption of the complexes to nitrocellulose filters; and equilibrium dialysis. The complex formation is Mg^{2+} dependent at low salt concentrations and becomes Mg^{2+} independent at an ionic strength greater than 90 mM. Oligouridylates of increasing chain length reach an optimal K_A of $3.3 \cdot 10^7$ M^{-1} at a chain length of n = 13-14. Protein S1 contains one binding site for long chain oligouridylates, such as U_{12}, and the standard-free-energy change of binding caused by one pU increment is 0.41 kcal/mol, when n varies between five and fourteen. Complex formation is insensitive to the capacity of the homopolynucleotide bases to form hydrogen bonds. Homopolynucleotides, however, showing a $T_m < 25^o$ in the buffer system used show an increased affinity for S1 compared to poly(A) and poly(C) ($T_m > 40^o$). The data are discussed with respect to the proposed binding of protein S1 to the 3'terminal end of the 16S RNA.

INTRODUCTION

The ribosomal protein S1 has gained recent scientific attention because it is unique in its physical properties and its function in protein biosynthesis as compared to the other ribosomal proteins |1|. Further interest in protein S1 has been stimulated because of its role in the initiation of Qβ RNA transcription |2|. Protein S1 as a host contributed subunit of Qβ replicase is probably responsible for the replication of the plus strand template and the translational repression activity of the replicase |3,4|.

Protein S1 behaves as a 10:1 prolate ellipsoid approximately 220 Å long with a molecular weight of 65,000 and a pI-value of 4.6 |1|. It contains 26% acidic amino acids, 8.2% lysine and 5.5% arginine and a noticeably high content of hy-

drophobic amino acids |5|. The association of S1 in the bound state to the 30S subunit is such that it freely exchanges with S1 free in solution and between subunits |1|. S1 is easily removed from 30S ribosomes by either low salt (1 mM Tris-HCl, pH 7.6) or high salt (1 M NH_4Cl) treatment |6,7|. It is routinely identified by its capacity to form a complex with $|^3H|$-polyuridylate which is retained on a nitrocellulose filter |8|, or by its requirement in the poly(U) dependent poly(Phe) synthesis |9|.

Protein S1 is indispensable for the translation of phage RNA |10|. It is thought to be involved in the initiation of protein synthesis, especially in the unfolding and binding of phage RNA |11|. This interpretation of S1 function is supported by the ability of S1 to change the secondary structure of single and double stranded nucleic acids |12,13| and by the fact that translation of formaldehyde treated i. e. unfolded MS2 RNA, does not require protein S1 |11|. Recent results have shown that protein S1 and initiation factors stimulate ribosome binding to the initiation region of the coat and replicase cistrons of R17 RNA to a greater extent than to the initiation site of the A protein cistron |14|. The initiation site of the A protein cistron, however, has the longest oligonucleotide complementary to the 3'end of the 16S RNA |15|.

Proton magnetic resonance spectra of the E3 RNA show that this 16S RNA fragment exists in a looped conformation with a single stranded 3'-terminal dodecanucleotide $AUCACCUCCUUA_{OH}$ |16|. Dahlberg and Dahlberg have presented experimental evidence that protein S1 binds to that dodecamer |17|, thereby making this region accessible for hydrogen bonding to the ribosome binding sites in phage RNA. According to this model it is assumed that protein S1 binds to the phosphate backbone of 16S RNA exposing the base moieties for a Watson-Crick type double strand formation with the intercistronic region of a mRNA. Therefore, it should be expected that complex formation between nucleic acids and protein S1 is related to the number of phosphate groups i. e. the chain length and does not primarily depend on the base moieties of the RNA.

In this report we present evidence that the association

constant increases with the chain length n of the oligonucleotide and remains constant, in the case of oligouridylate, when n exceeds 13-14. The dependence of complex formation on the properties of the bases in the homopolynucleotides stresses the importance of the stability of their secondary structure rather than their ability to form hydrogen bonds. Together, these findings are taken as strong evidence that polynucleotide binding to S1 is determined by the interaction of the protein with the phosphate backbone of the polynucleotide. The findings are discussed with respect to the molecular model of S1 function as proposed by Dahlberg and Dahlberg |17|.

MATERIALS AND METHODS

Escherichia coli MRE 600 and Micrococcus luteus were obtained from Merck, Darmstadt and N-ethyl|2.3-^{14}C|maleimide (|^{14}C|-NEM) (spec.act. 4 Ci/mol) from Amersham Buchler, Braunschweig. Selectron BA 85 filters were purchased from Schleicher und Schüll, Dassel and GF/A filters from Whatman, London. Membranes for equilibrium dialysis were obtained from Rhône Poulenc, Paris. Polynucleotide phosphorylase was prepared according to the method of Schetters et al. |18|.

Preparation of the homopolynucleotides and of $(Up)_nU$ oligomers.

2.5 ml of the following reaction mixture for the synthesis of the polynucleotides were incubated at 37°C for 3 h: 25 mM Tris-HCl, pH 9.5, 220 mM KCl, 2.5 mM $MgCl_2$, 36 µmol nucleotide-5'-diphosphate, 300 µg polynucleotide phosphorylase (150 U/ml), and 0.1 mCi of the nucleoside 5'-diphosphate in the case of radioactively labeled polynucleotides. The reaction was stopped by heating the mixture at 80°C for 5 min. The enzyme was removed by extracting three times with phenol followed by four extraction with ether. The polymer was purified by Sephadex G-50 column chromatography (1 cm x 50 cm). Poly(m^3U) was synthesized by methylating poly(U) according to Pochon and Michelson |19| and poly($h_2^{5,6}$U) by reduction of the same compound with $NaBH_4$ and UV irradiation |20|. $(Up)_nU$ and $(Ap)_nA$ oligomers with a chain length of 3-16 nucleotides were prepared by polynucleotide phosphorylase catalyzed polymerisation following standard procedures |21,22|. Chain lengths were determined with the aid of a nucleoside analyzer |23|.

Preparation of homopolynucleotides tritiated in the 3'-terminal nucleoside.

Because the radioactive nucleoside 5'-diphosphates were not available, the homopolynucleotides were labeled in the terminal cis-diol end group. This method involved the reaction of an aqueous homopolynucleotide solution with sodium metaperiodate and subsequent reduction of the resulting dialdehyde with $|^{3}H|$-$NaBH_4$ |24,25|. Tritiated homopolynucleotides were purified by Sephadex G-50 column chromatography.

Isolation of protein S1.

70S ribosomes were prepared from E. coli MRE 600 according to Staehelin and Maglott |26| and subunits were obtained by zonal centrifugation |27|. Protein S1 was isolated from the 30S subunits by the method of Tal et al. |5|. The protein was dissolved in 25 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 50 mM KCl up to a concentration of 10 mg/ml and stored at -20°C.

Reaction of $|^{14}C|$-NEM with protein S1 in the presence of homopolynucleotides.

The reaction mixture contained in a total volume of 235 µl: 220 µl binding buffer (10 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$), 200 pmol protein S1, 10 nmol $|^{14}C|$-NEM and homopolynucleotide as indicated in the legends to the figures. The homopolynucleotides were preincubated for 3 min with the protein prior to the addition of NEM. After standing for 24 h at 4°C the 7 ml test tubes were filled with ice cold binding buffer and the solutions were filtered through Selectron BA 85 filters. The filters were washed once with the same volume of binding buffer. The radioactivity remaining on the filters was determined in 2 ml toluene 0.4% 2.5 diphenyloxazol. The counting efficiency was 80%. Under these conditions 200 ± 10 pmol of NEM were bound to 200 pmol S1 in the absence of homopolynucleotides.

Binding of labeled homopolynucleotides to protein S1 in the presence of NEM.

The reaction mixture contained in a total of 235 µl: 220 µl binding buffer, 200 pmol protein S1, 10 nmol NEM and homopolynucleotides as indicated in the legends of the figures. The subsequent procedure was the same as in the NEM inhibition assay except that alkaline treated (0.5 NaOH, 25°C 1 h) nitrocellu-

lose filters were used.

Equilibrium dialysis.

The binding of oligo(^{3}H|U) and oligo(^{3}H|A) of defined chain length to protein S1 was examined by equilibrium dialysis at 4°C. The dialysis chambers contained in a total volume of 200 µl: 10 mM Tris-HCl, pH 7.6, 10 mM Mg^{2+}, 200 mM NaCl, and half saturating oligonucleotide concentrations which were calculated from previous experiments. One chamber contained 75 pmol of protein S1. The equilibrium endpoint was determined in separate experiments for each oligonucleotide. Four 20 µl aliquots were removed from each chamber and counted separately on GF/A filters. 200 mM NaCl was used to minimize the Donnan effect. When the Mg^{2+} dependence of the U_{12} binding to protein S1 was measured the opposite chamber to the S1 chamber contained 75 pmol bovine serum albumin which showed no binding affinity for U_{12}.

RESULTS

Purification and purity of protein S1.

Protein S1 was isolated according to procedures published by Tal et al. |5|. However, the crude S1 extract obtained by dialysis of 30S subunits against 1 mM Tris-HCl, pH 7.6, showed only a A_{280}/A_{260} ratio of about 1.0 and further purification by DEAE cellulose chromatography was necessary. As shown in figure 1, peak 3 contained S1 with a A_{280}/A_{260} ratio of 1.55,

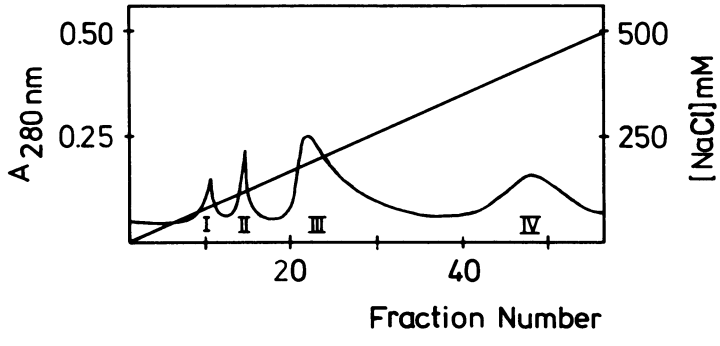

Fig. 1 DEAE cellulose chromatography of the crude S1 extract. Column dimension: 2 cm x 8 cm, flow rate: 1.3 ml/min, fraction volume: 5.4 ml

which conforms to the value reported by Laughera et al. |1|. The material of peaks 1 and 2 was identified as oligonucleotides and peak 4 as a S1•RNA complex. 10,000 A_{260}-units 30S ribosomes (620 nmol) yielded 21 mg protein S1 (320 nmol) which showed a single band on SDS gel electrophoresis (50 µg/gel). 1 mg S1 gave an optical density reading of 0.75 at 280 nm |1|. The binding capacity was 20-25 pmol of polyuridylate (average chain length 80) per 40 pmol of S1 as determined by the nitrocellulose filter assay. An exact stochiometry of 1:1 was obtained with U_{12} using the equilibrium dialysis technique. The number of sulfhydryl groups was two when S1 was titrated with $|^{14}C|$-NEM in the presence of 6 M guanidinium hydrochloride (results not shown). The S1 preparation showed no nuclease activity upon incubation with $|^{3}H|U_{12}$ or $|^{3}H|A_{12}$ for 24 h at 4°.

Binding of oligo- or polynucleotides to protein S1.

Three methods were used to study the complex formation between S1 and oligo- or polynucleotides:

1. Inhibition of the covalent binding of $|^{14}C|$-NEM to protein S1 in the presence of oligo- or polynucleotides.
2. Adsorption of S1•poly(N) complexes to alkaline treated nitrocellulose filters.
3. Equilibrium dialysis with a membrane highly permeable to oligonucleotides |28|.

Each of the three methods show certain advantages and limitations, which are dealt with in the discussion section.

Mg^{2+}-dependence of S1•poly(N) complex formation.

Mg^{2+}-dependence of complex formation was assayed using the three methods, and identical results were obtained. The binding of polynucleotides is Mg^{2+} dependent at low ionic strength (Figure 2). There was no difference in the Mg^{2+} optimum (10 mM) if poly(U) is replaced by poly(C) (data not shown). No Mg^{2+}-dependence is found at an ionic strength above 80 mM NaCl (Figure 3). This shows that Mg^{2+} effects oligonucleotide binding specifically and that Mg^{2+} can be replaced by monovalent ions at much higher ionic strengths only. Whether Mg^{2+} exerts its function on the polynucleotide structure, on the protein conformation or in the protein nucleic acid interaction can not be discerned from these experiments.

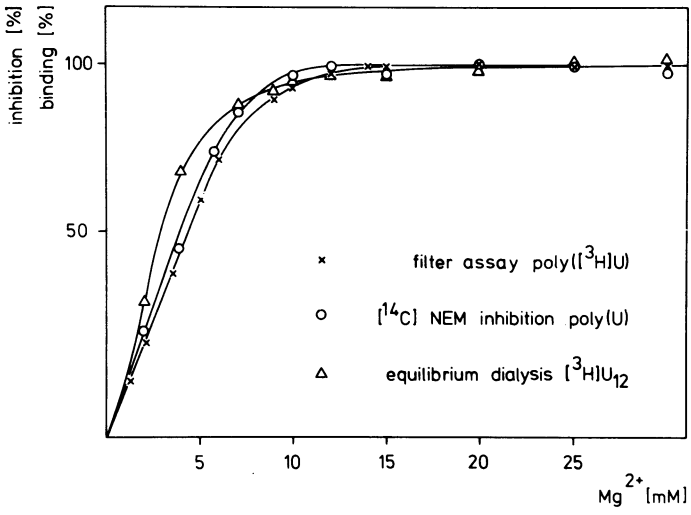

Fig. 2 Mg^{2+}-dependence of complex formation between protein S1 and poly(U) or U_{12} in 10 mM Tris-HCl, pH 7.6. For this measurement three methods i. e. adsorption to nitrocellulose filters, inhibition of the NEM reaction, and equilibrium dialysis were used.

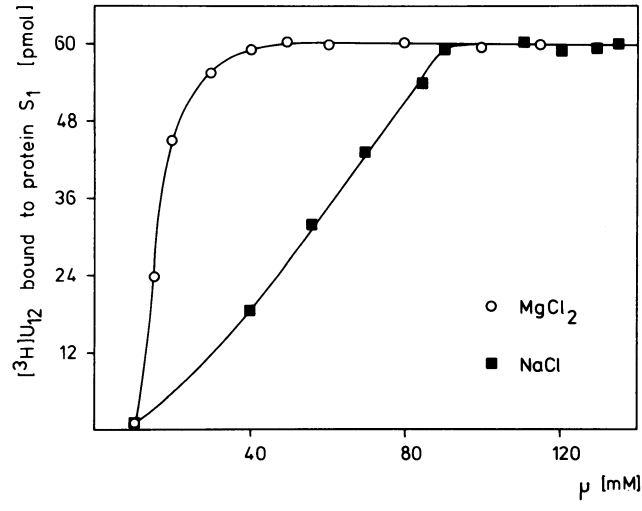

Fig. 3 Ionic strength dependence of complex formation between $|^3H|U_{12}$ (116 pmol) and S1 (75 pmol) with either $MgCl_2$ or NaCl as examined by equilibrium dialysis (10 mM Tris-HCl, pH 7.6, 75 pmol BSA)

Binding of oligouridylates with increasing chain length to protein S1.

Chain length dependent complex formation between S1 and oligouridylates n < 15 was investigated by equilibrium dialysis and for n > 15 by the filter assay because the membranes are impermeable for polynucleotides.

The association constants for the S1 oligouridylate complexes and the number of oligonucleotides bound per S1 were determined by means of a Scatchard plot (Table 1).

chain length n	K_A $\lvert M^{-1} \rvert$	number of oligomer bound per S1
5	$4.9 \cdot 10^4$	2.50
7	$2.3 \cdot 10^5$	0.93
9	$1.1 \cdot 10^6$	0.94
12	$9.0 \cdot 10^6$	1.05
14	$2.5 \cdot 10^7$	0.90
$\overline{30}$	$3.3 \cdot 10^7$	0.85
$\overline{49}$	$3.8 \cdot 10^7$	0.71
$\overline{80}$	$4.0 \cdot 10^7$	0.62

Tab. 1 Association constants for the $S1 \cdot U_n$ complex formation. Data were obtained from a Scatchard plot. The Scatchard plot was calculated by linear regression from sorted data points with

$$f_i = \frac{1}{\Delta\bar{\nu} \cdot \Delta(\bar{\nu}/\bar{L})}.$$

Using U_5 more than one molecule was bound to S1, whereas from U_7 to U_{14} the number of ligands bound is reduced to one. For oligouridylates with a chain length of $n >> 14$ more than one molecule S1 binds to the oligomer. When ln K_D was plotted versus the chain length of the oligonucleotides Figure 4 was obtained showing two straight lines intersecting at ln K_D = -17.3 and n = 13.6. This finding strongly suggests that protein S1 contains one binding site for oligouridylates, which accommodates about 13-14 nucleotides with an association constant of $3.3 \cdot 10^7$ M^{-1}. If the chain length is much shorter than 14 this

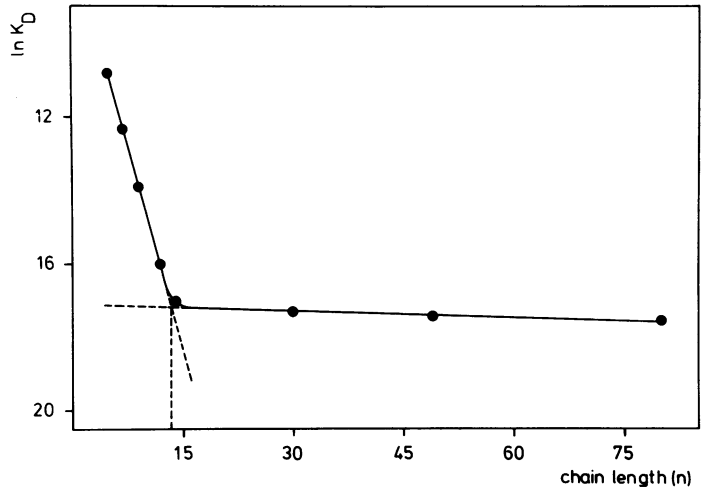

Fig. 4 Plot of ln K_D versus the chain length n of the oligouridylates

site can be saturated with more than one oligonucleotide which renders a decrease in the association constant as was determined with U_5.

A standard free enthalpy change of ΔG^o = 0.41 kcal/mol was calculated for one pU increment from the slope of the curve between n = 5 and n = 13. In the range of n < 5 the extension of the curve in Figure 4 should be nonlinear, since at n = 0 K_A should be 0.

We were able to show that NEM inhibition takes place simultaneously with the binding of oligouridylates, e. g. $U_{\overline{80}}$, to S1 (Figure 5). Therefore, we repeated the experiment of chain length dependence of complex formation by using the NEM inhibition technique (Figure 6).

It was found that oligouridylates of decreasing chain length which show decreasing K_A-values with S1 (Table 1) are also reduced in the ability to inhibit the NEM reaction. Thus, we maintain that binding of nucleic acids to S1 effects NEM inhibition and that the amount of inhibition is regulated by the complex binding affinity for S1. Moreover, the results of Figures 2 and 5 show the NEM inhibition technique to be valid. Although the NEM inhibition technique is an indirect method for

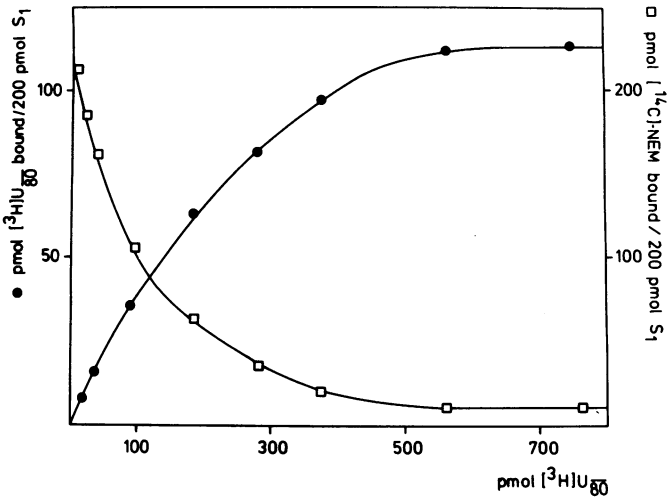

Fig. 5 Binding of $|^{3}H|U_{\overline{80}}$ to S1 in the presence of a 50 molar excess of $|^{14}C|$-NEM

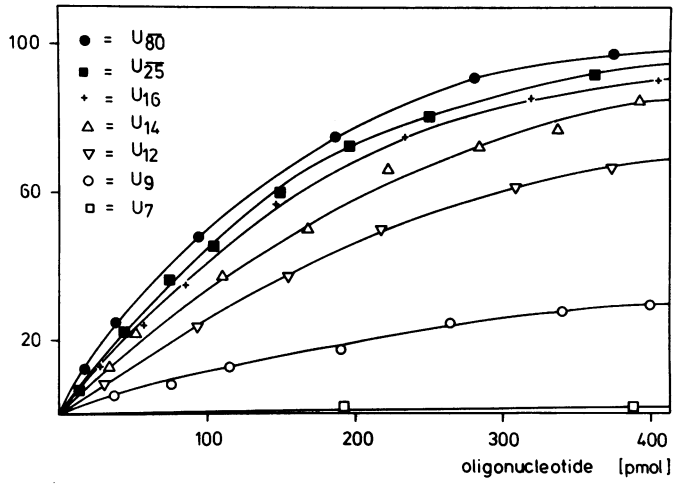

Fig. 6 Inhibition of the $|^{14}C|$-NEM reaction with protein S1 in the presence of increasing amounts of oligouridylates, chain length U_7 to U_{16}, and average chain length $U_{\overline{25}}$ and $U_{\overline{80}}$. 100% inhibition refers to the residual amount of $|^{14}C|$-NEM bound to S1 for saturating amounts of $U_{\overline{80}}$.

following complex formation and its mechanism is unknown, we maintain that this method is reliable for determining whether a nucleic acid forms a complex with S1.

Binding of polynucleotides with different base moieties to protein S1.

As was shown above, the NEM inhibition test proved to be a useful method for following the complex formation. If polynucleotides with different base moieties are to be examined, method 1 is preferred over method 2 and 3 for two reasons: Polynucleotides cannot pass the dialysis membrane and compounds like poly(A) and poly(C) tend to adhere to alkaline treated nitrocellulose filters in the presence of Mg^{2+} or high salt concentrations.

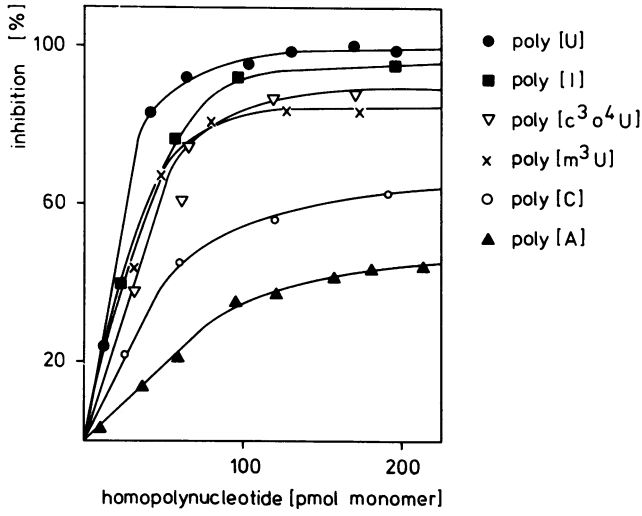

Fig. 7 Inhibition of the $|^{14}C|$-NEM reaction with protein S1 in the presence of increasing amounts of homopolynucleotides.

Poly(U), poly(I), poly(m^3U), and poly(c^3o^4U) all display a similar degree of NEM inhibition (Figure 7). Thus, it appears that the base portion of the polynucleotide plays no direct role in complex formation |8|. Poly(U) and poly(I) show comparable binding, so that a preference for pyrimidine nucleotides can be ruled out. Hydrogen bonds from the protein to the

N(3) and C = O(4) are unlikely as shown by poly(m^3U) and poly(c^3o^4U). There is no evidence for hydrophobic interactions as a major contribution to complex stability since poly($h_2^{5.6}U$) binds comparable to poly(U) (see Figure 8). All of these homopolynucleotides have a secondary structure of low thermal stability in the buffer used (T_m < 25°C). Poly(A) (T_m > 50°C) and poly(C) (T_m > 40°C) on the other hand, which have a more thermostable secondary structure, clearly show a reduced affinity for the protein.

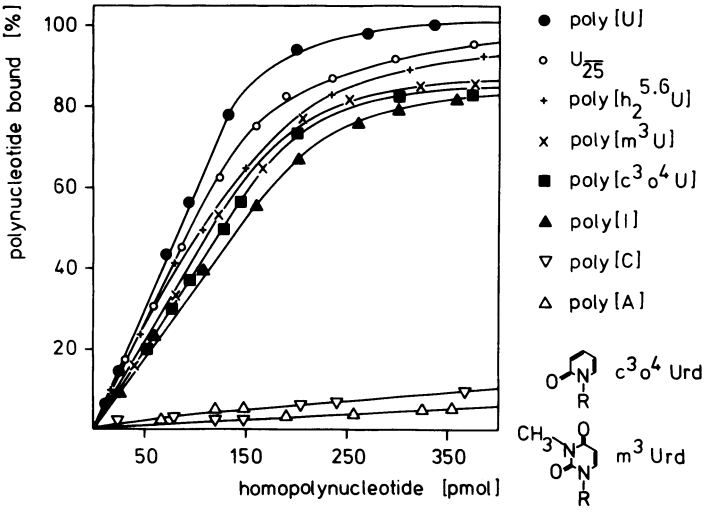

Fig. 8 Binding of homopolynucleotides tritiated in the cis diol end group to protein S1.

Since the NEM inhibition test is an indirect method, binding of end group labeled homopolynucleotides (see methods) to S1 was examined by the adsorption to nitrocellulose filters in the presence of unlabeled NEM (Figure 8). The data are in good agreement with the results from the NEM inhibition test. Homopolynucleotides with a low T_m-value have a greater affinity to protein S1 compared to poly(A) and poly(C).

Comparison of oligouridylates and oligoadenylates in complex formation.

In order to present further evidence that nucleic acids by the thermostability of their secondary structure might influence the complex formation with S1 $|^3H|$-oligouridylates and

$|^3H|$-oligoadenylates of defined chain length were compared in their binding properties to S1 (equilibrium dialysis). These compounds were selected because it is well known from the T_m-values of both types of oligonucleotides that oligoadenylic acids have a more rigid secondary structure compared to oligouridylates. The association constants of oligo($|^3H|U$) and oligo($|^3H|A$) are listed in Table 2. If identical chain lengths are compared, the association constant was roughly 10fold lower in the case of oligo($|^3H|A$).

chain length n	K_A $\|^3H\|A_n$ M^{-1}	K_A $\|^3H\|U_n$ M^{-1}
5	$<5.0 \cdot 10^3$*	$4.9 \cdot 10^4$
7	$4.7 \cdot 10^4$	$2.3 \cdot 10^5$
9	$2.0 \cdot 10^5$	$1.1 \cdot 10^6$
12	$6.6 \cdot 10^5$	$9.0 \cdot 10^6$
14	$2.6 \cdot 10^6$	$2.5 \cdot 10^7$

Tab. 2 Comparison of the K_A-values of $|^3H|A_n$ and $|^3H|U_n$ as determined by equilibrium dialysis.
*This value gives only an approximation because of the experimental error (see discussion)

DISCUSSION

The protein S1, as purified by DEAE cellulose chromatography, was virtually free of nucleases. This is an absolute prerequisite for equilibrium dialysis experiments requiring long incubation periods. The final peak from the column, which contained the S1•RNA complex, could be dissociated into S1 and an oligonucleotide by 7 M urea. Complex formation between oligo- and polynucleotides was followed by the techniques listed in the results. Obviously, equilibrium dialysis is the most reliable method for determining the association constant of oligonucleotides. It is, however, limited to oligonucleotides $n < 15$ because oligonucleotides with longer chain length will not pass through the membrane within an acceptable period of time. Furthermore, it is difficult to measure binding constants $<10^4$ M^{-1}

because the differences in cpm in both chambers are within the limit of experimental error. Although the adsorption of the complexes to alkaline treated nitrocellulose filters is the classical and rapid method, the interpretation of the results is ambiguous because the required assumptions about the kinetics of dissociation might not be justified. Further experimental problems arise due to the adsorption of some oligonucleotides, such as poly(A), to the filters. We have presented experimental evidence that complex formation can be followed by the NEM inhibition technique. We assume that the mechanism of inhibition is always the same in spite of the substrate used and that inhibition depends on the complex binding constant only. Although this assumption might appear unjustified our results support the validity of the above statement.

Complex formation was found to be Mg^{2+} dependent at low salt concentrations (10 mM Tris-HCl, pH 7.6). It appears to be a specific Mg^{2+} ion concentration dependency because the optimal Mg^{2+} concentration of 10 mM for complex formation is only replaceable by an ionic strength of 80 mM NaCl in the case of monovalent ions.

With U_5 to U_{12} one finds a linear increment per pU residue added. With shorter chain length a "nucleation" effect may exist, similar to double strand formation in nucleic acids. These data in combination with the lack of any direct base specificity would point towards electrostatic interaction as the sole source of complex stability. Since, however, complex formation is not reduced in the presence of high salt concentrations (Figure 2) and the blocking of the lysyl ε-ammonium group does not interfere with S1 function |29|, this interpretation appears to be oversimplified.

We have shown with the use of base modified homopolynucleotides that no direct base preference exists. There is still the possibility, that if bound to the 3'end of the 16S RNA, S1 is sequence specific. Since, however, E3RNA is displaced by polyuridylate from its complex with S1, this seems not to be the case |17|.

From the association constant for S1•U_{13} formation a standard-free-enthalpy change of $\Delta G^{o} = -9.8$ kcal/mol can be calcu-

lated. If one assumes that U_{13} or poly(U) has to be reoriented from its native structure at 4°C and 15 mM Mg^{2+} |30|, to become an optimal ligand for protein S1, this enthalpy change should be sufficient in the case of a polynucleotide with a low T_m-value. If a polynucleotide with a highly thermostable secondary structure like poly(A) or poly(C) is used, the free energy of binding might be insufficient to counter balance the reaction enthalpy of melting. If this is correct, the decreased association constant of oligo(A) as compared to oligo(U) might be explained by the assumption that the former cannot adapt its secondary structure in order to permit optimal phosphate backbone-S1 interaction. Therefore, not all of the phosphate groups of oligo(A), only the exposed ones, would stabalize the binding to protein S1. This model reduces the base specificity of S1 binding to the flexability of the oligonucleotide structure and explains the higher affinity of oligouridylates as compared to oligoadenylates for S1.

In the ribosomal system S1 could stabilize, in combination with initiation factor IF-3, the 3'end of the 16S RNA in a conformation optimized for double strand formation with the intercistronic region of a mRNA. It seems to be not accidental that a chain length of 12-14 gives optimal complex formation and that the dodecanucleotide contains preferentially pyrimidines. In general we believe that the data presented, strongly support the model of S1 function as proposed by Dahlberg and Dahlberg |17|.

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