CHARACTERIZATION OF EXTENDED SEQUENCES AROUND THE COAT AND REPLICASE CISTRON RIBOSOME BINDING SITES IN PHAGE Qβ RNA

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1. Introduction

The nucleotide sequences around the ribosome binding sites in Qβ and R17 RNA [1, 2] first indicated the presence of non-translated 'spacers' separating the different phage cistrons. In phages R17 and MS2 an untranslated region of 36 nucleotides lies between the coat and replicase cistrons [3, 4], and in Qβ RNA the first 61 nucleotides at the 5'-end do not code for amino acids [5]. These intercistronic areas have been implicated as containing special oligonucleotide sequences or structures [6, 7] that allow ribosomes to distinguish the AUG initiator codons from all other AUG triplets. Such similarities as do exist in the sequences preceding the different phage cistrons do not, however, appear to define the reason for this specificity, and for this reason we were interested in obtaining longer fragments from the initiation sites than have previously been isolated, taking advantage of the lowered susceptibility of base-paired G residues to T_1 RNAase digestion at 0° in a buffer of fairly high ionic strength [8]. Until now, the limited sequence of nucleotides known around ribosome binding sites has made it difficult [6] to make any deductions regarding secondary structure.

In this paper we report the isolation of several T_1 RNAase-resistant Qβ RNA fragments (up to about 5 S in size) from the coat and replicase cistron ribosome binding sites. One is 66 nucleotides in length and contains neither an AUG nor a GUG codon. A further fragment, 82–83 nucleotides in length, contains the replicase cistron ribosome binding site [7].

2. Materials and methods

^{32}P-labelled Qβ RNA (10 A_{260} units; 5×10^8 cpm) was bound to *E. coli* MRE 600 ribosomes in the presence of unfractionated fMet-$tRNA_F$ and GTP under conditions that are described in detail elsewhere [1], and the initiation complex was identified in the 70 S region of a 4–20% sucrose gradient. The fractions containing the 70 S ribosomes were combined, centrifuged at 150,000 *g* for 3 hr, and the ribosomes resuspended in 1 ml ribosome buffer (50 mM Tris-HCl pH 7.8, 50 mM NH_4Cl, 5 mM $Mg(CH_3COO)_2$ and 6 mM 2-mercaptoethanol). 30 μg T_1 RNAase was added and incubated for 18 hr at 0°. The ribosomes with the associated radioactive Qβ RNA fragments were again resolved from the unbound material by sucrose gradient centrifugation and the total RNA extracted, precipitated in ethanol and fractionated by electrophoresis through a 12% polyacrylamide gel slab (see legend to fig. 1). The radioactive bands were located by autoradiography and RNA extracted from each three times over a 4 hr period with a total of 5 ml H_2O and 80 μg carrier RNA. The RNA was isolated by ethanol precipitation, dissolved in a small volume of water and dried.

3. Results

Fig. 1 shows the labelled RNA protected in the 70 S complex to be heterogeneous. Half the RNA extracted from each major gel band was digested with

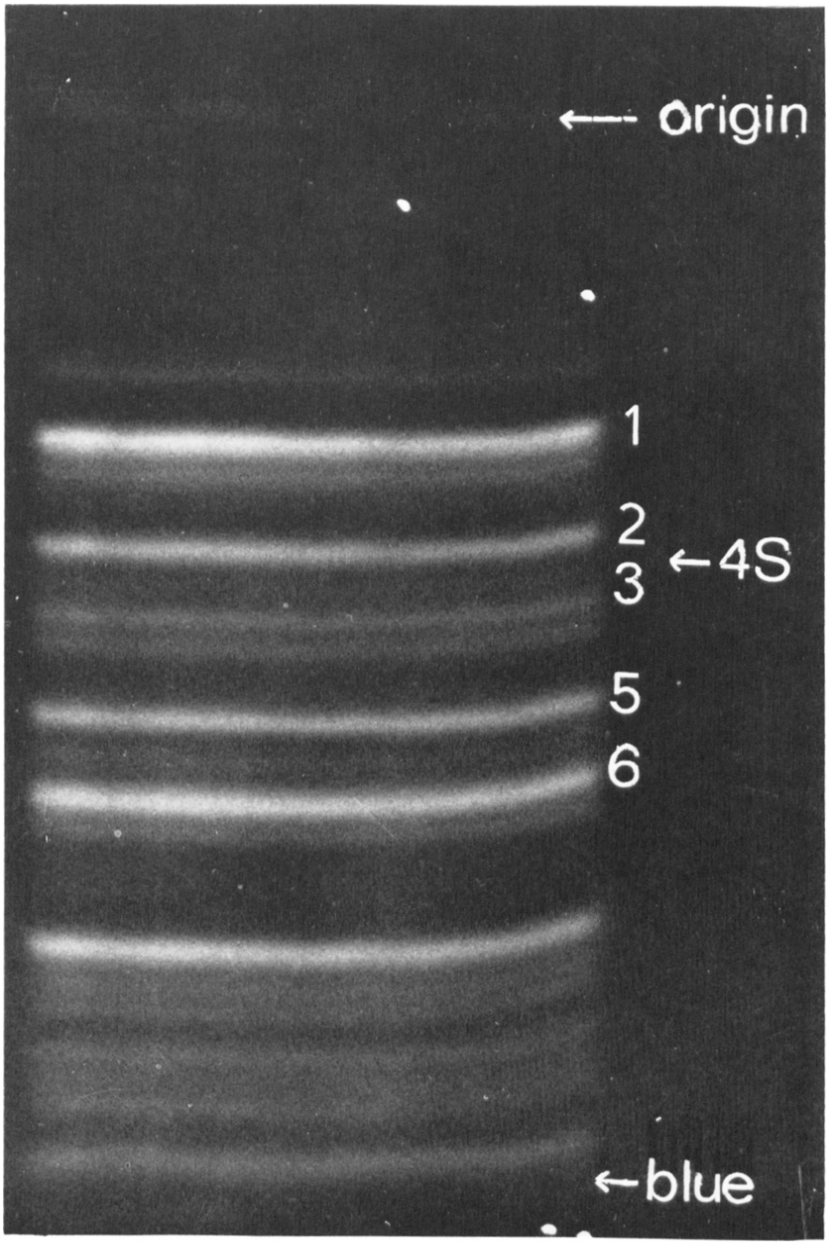

Fig. 1. Gel electrophoresis of RNA isolated from 70 S–Qβ RNA complex. The RNA from the 70 S initiation complex was dissolved in 10% sucrose and a trace of bromophenol blue, and layered on a 12% polyacrylamide gel in slots measuring 1 × 7 × 8 mm and electrophoresed at 400 V for 6 hr in a buffer containing 0.1 M Tris pH 8.3; 0.0025 M EDTA and 0.1 M boric acid. The gel was autoradiographed, and the radioactive RNA extracted from the areas corresponding to the numbered bands (see Materials and methods for details).

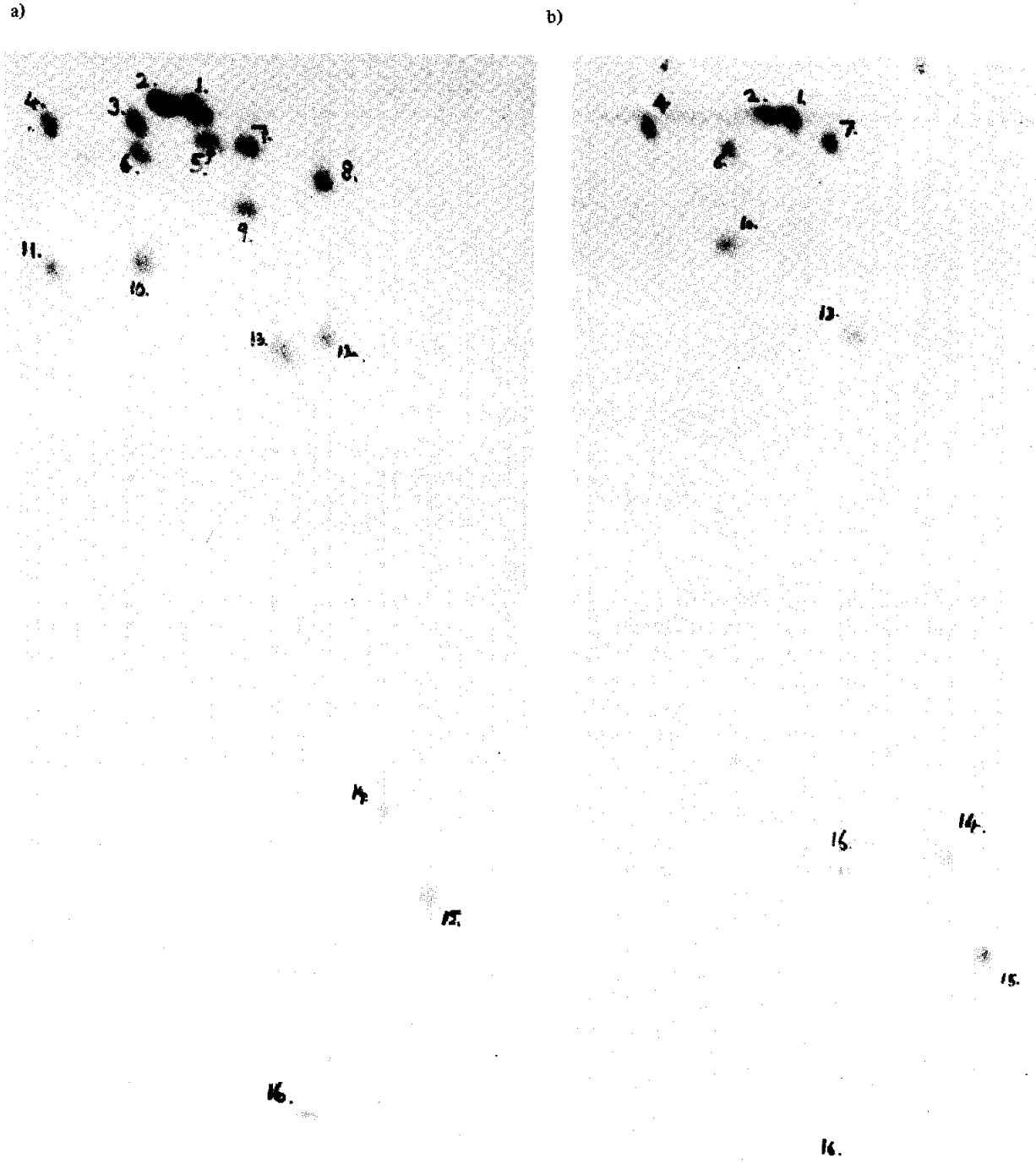

Fig. 2. T_1 RNAase fingerprints of Qβ initiator fragments. a) Fragment 1; b) Fragment 5. The T_1 oligonucleotides missing in fragment 5 include AUCAUG, which contains the initiator codon of the coat cistron. The T_1 RNAase fingerprint of fragment 6 (not shown) contains the oligonucleotides present in fragment 1 that are absent in fragment 5.

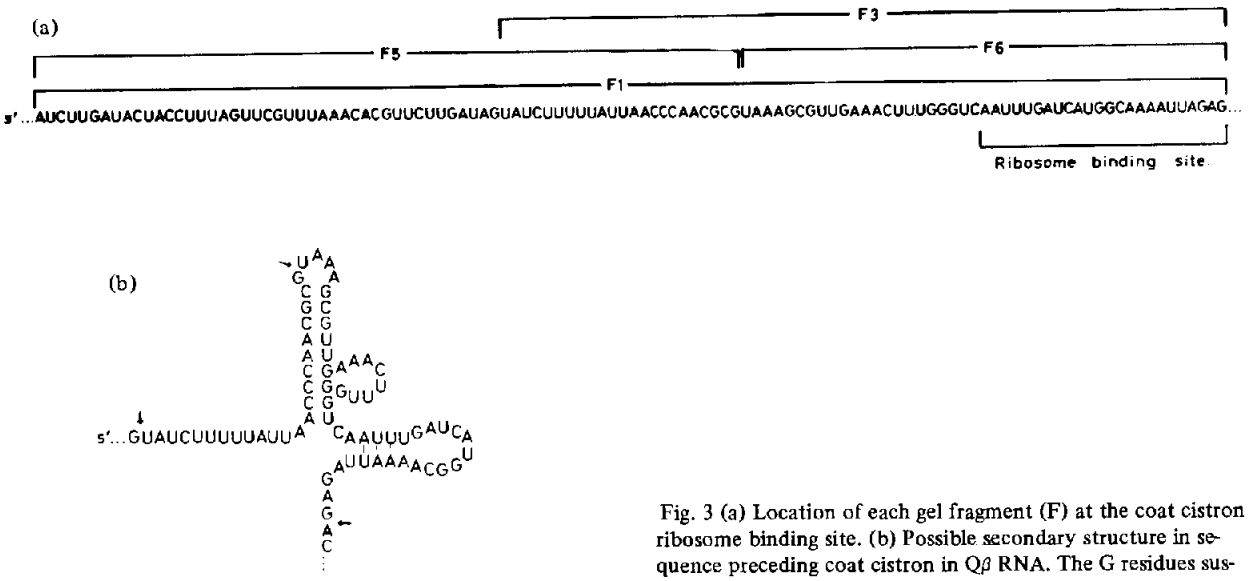

Fig. 3 (a) Location of each gel fragment (F) at the coat cistron ribosome binding site. (b) Possible secondary structure in sequence preceding coat cistron in Qβ RNA. The G residues susceptible to T_1 RNAase digestion are indicated by arrows.

pancreatic RNAase, and half digested with T_1 RNAase. The resultant oligonucleotides were fractionated, counted and identified by established methods [8, 9]. Table 1 gives the oligonucleotides present in the five fragments analysed, and it is clear that fragments 5 and 6 result from a split in fragment 1 (see also fig. 2). The presence of fragment 5 at first seemed surprising as it lies a considerable distance from the start of the coat cistron (fig. 3a). A knowledge of the sequence of 97 nucleotides preceding the coat cistron [10] suggests that fragment 5 arises by a 'hidden break' [3] at the top of a loop of secondary structure in intact Qβ RNA (fig. 3b). The H-bonding at the surface of the ribosome holds the two pieces together until disrupted following phenol extraction of the RNA from the 70 S complex. What justification is there for writing such a structure? Firstly, base-pairing can be maximized to give a thermodynamically stable structure [11]. Secondly, none of the G residues involved in base-pairing in the proposed loop are attacked by T_1 RNAase [4, 10] under the conditions used. It is difficult to otherwise explain the origin of fragment 5, unless it represents a hirtherto unknown earlier event in initiation. Part of the sequence preceding the coat cistron appears to be repeated (fig. 4). Duplications (and deletions) have been shown to exist in other regions of Qβ RNA and in related phages [12].

Fragment 2, 82–83 nucleotides in length, includes the replicase initiation site (table 1), since it contains all the oligonucleotides which form the sequence of 31 nucleotides already known [7]. The sequence of fragment 12 is at present being determined and will be reported in a later paper.

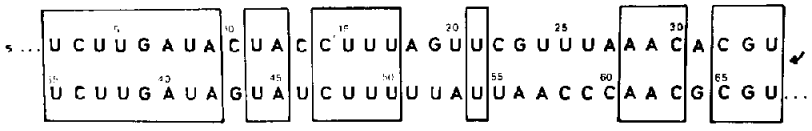

Fig. 4. Partially repeated sequence preceding coat cistron of Qβ RNA. Nucleotides 2 to 34 are shown directly above nucleotides 35 to 67. The boxed in residues show the extent of homology.

Table 1

Relative molar amounts of different oligonucleotides produced by pancreatic and T_1 RNAase digestion of various Qβ initiator fragments.

a)

Sequence	Pancreatic RNAase: Yield of oligonucleotide* per fragment			Spot no. (fig. 2)	Sequence	T_1 RNAase: Yield of oligonucleotide* per fragment			
	1.	5.	6.			1.	3.	5.	6.
GGGU	1.0	–	1.0	1	AUACUACCUUUAG	1.0	–	1.1	–
AGAG	0.9	–	1.0	2	UAUCUUUUUAUUAACCCAACG	0.6	0.5	0.5	–
AAAAU	1.1	–	1.0	3	UCAAUUUG	1.0	1.0	–	1.0
AGU	2.4	2.3	–	4	UUCUUG	1.1	–	1.2	–
GAU	3.2	2.2	1.5	5	AAACUUUG	0.9	1.0	–	1.1
GGC	1.1	–	0.9	6	AUCUUG	1.1	–	0.8	–
AAAGC	0.9	–	1.1	7	AAACACUUUG	1.0	–	1.0	–
GAAAC	1.0	–	1.0	8	CAAAAUUAG	0.6	0.8	–	0.8
GU	4.7	2.9	2.2	9	AUCAUG	1.2	1.1	–	0.8
AAU	1.3	–	1.3	10	UUCG	1.7	–	1.2	–
AAAC	1.0	1.0	–	11	UUG	1.5	1.1	–	1.0
AU	4.7	4.1	2.3	12	UAAAG	0.9	0.9	–	0.9
GC	1.1	1.2	–	13	AUAG	1.3	–	0.9	–
AAC	2.2	2.5	–	14	AG	1.7	1.2	–	1.3
AC	3.0	3.5	–	15	CG	3.2	2.5	1.6	1.6
U	~25	~19	~12	16	G	4.0	3.6	1.2	2.9

* Relative to mean molar yield of GGGU, AAAC and GAAAC or AAACUUUG and AAACACUUUG.

a: Coat initiation site; b: replicase initiation site (fragment 2).

b)

Sequence	Pancreatic RNAase: Found yield**	Structure	T_1 RNAase: Found yield**
AAGGAU	1.0	$[C_3,AAC,U_4]AG$	0.8
GAAAU	0.9	[CAU,CU,U]CG	1.0
GAGC	1.0	UAACUAAG + CACAAUUG	1.6
AAGAC	1.0	UCUAAG	0.8
GU	2.4	AAAUG	0.9
GAAC	1.0	AUG	1.1
AAG	0.9	CAUG	1.0
AAU	1.2	AACACAAG	0.6
AGC	2.0	ACAG	0.9
AU	2.3	AG	1.3
GC	5.9	CCG	2.0
AAC	2.0	CG	4.0
AC	2.2	G	1.3
U	8.3		
C	7.8		

** Relative to mean molar yield of AAGGAU and GAGC or [CAU,CU,U]CG, UCUAAG and AAAUG.

4. Discussion

The presence of fragment 5 in the 70 S initiation complex suggests the existence of a particular secondary structure at the coat site under conditions of no protein synthesis. In binding at the correct site, the ribosome may be influenced by local secondary structure in addition to the nucleotide sequence close to the AUG initiation triplet. However, the observation that, even after degradation of Qβ RNA by partial alkaline hydrolysis to fragments of average molecular weight less than 5 S, ribosome binding only occurs at the true initiation sites [7] implies that regions of secondary structure involving the juxtaposition of widely separated primary sequences are not involved in ribosome recognition, though this feature plays an important role in masking potential binding sites in the intact molecule [2, 4, 13].

The approach described here provides a means of obtaining extended nucleotide sequences and information about the secondary structure of the mRNA in the vicinity of the ribosome binding site.

Acknowledgements

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References

[1] J. Hindley and D.H. Staples, Nature 224 (1969) 964.
[2] J.A. Steitz, Nature 224 (1969) 959.
[3] J.L. Nichols, Nature 225 (1970) 147.
[4] W. Min Jou, G. Haegeman, M. Ysebaert and W. Fiers, Nature 237 (1972) 82.
[5] D.H. Staples, J. Hindley, M.A. Billeter and C. Weissmann, Nature New Biology 234 (1971) 202.
[6] J.A. Steitz, Nature New Biology 236 (1972) 71.
[7] D.H. Staples and J. Hindley, Nature New Biology 234 (1971) 211.
[8] J.M. Adams, P.G.N. Jeppesen, F. Sanger and B.G. Barrell, Nature 223 (1969) 1009.
[9] F. Sanger, G.G. Brownlee and B.G. Barrell, J. Mol. Biol. 13 (1965) 373.
[10] H. Weber, M.A. Billeter, S. Kahane, C. Weissmann, J..Hindley and A. Porter, Nature New Biology 237 (1972) 166.
[11] I. Tinoco, O.C. Uhlenbeck and M.D. Levine, Nature 230 (1971) 362.
[12] J.M. Adams and S. Cory, Nature 227 (1970) 570.
[13] H.F. Lodish and H.D. Robertson, Cold Spring Harbor Symp. Quant. Biol. 34 (1969) 655.