Nucleic Acids Research

Volume 11 Number 21 1983 Nucleic Acids Research

The organization of the 7SL RNA in the signal recognition particle

Eckart D.Gundelfinger, Elke Krause, Marialuisa Melli and Bernhard Dobberstein

European Molecular Biology Laboratory, Postfach 10.2209, 6900 Heidelberg, FRG

Received 5 September 1983; Revised and Accepted 14 October 1983

ABSTRACT

Digestion of the signal recognition particle (SRP) of dog pancreas with micrococcal nuclease results in the stepwise cleavage of the 300 nucleotide 7SL RNA moiety producing five major fragments approximately 220 (1), 150 (2), 72 (3), 62 (4) and 45 (5) nucleotides long. The RNA molecule is initially cut once yielding fragments 1 and 3. Further degradation releases fragments 2, 4 and 5. The introduction of the first nick into the 7SL RNA does not alter the structure nor the function of the SRP. Further degradation of the RNA results in disruption and loss of activity of the particle.

The sequence of the RNA fragments shows that the nuclease causes discrete cuts in the RNA with minimal nibbling indicating that only few sites are accessible to the action of the enzyme. The five major products of nuclease digestion together span almost the entire length of the 7SL RNA. Nicking occurs mainly around the boundary region between the central S sequence and the flanking Alu sequences constituting the 7SL RNA (1). The S fragment is bound to the four largest polypeptides while the 5' and 3' Alu fragments are associated with the two smallest protein constituents of the SRP.

INTRODUCTION

The initial step in the translocation of proteins across membranes is mediated by at least two components: 1. The signal recognition particle, a soluble ribonucleoprotein containing at least six polypeptides of molecular weight 72, 68, 54, 19, 14 and 9 K (2) and one RNA molecule called 7SL RNA (1,3,4). 2. The docking protein which consists of a polypeptide of molecular weight 72 K, integrated in the membrane of the endoplasmic reticulum with a 60 K domain exposed on the cytoplasmic side (5-8).

After initiation of the synthesis of a secretory protein on a free ribosome and polymerization of 60-70 amino acids, the SRP interacts with the nascent polypeptide chain and arrests further synthesis until contact has been made with the docking protein. Completion of protein synthesis and translocation across the membranes can then proceed (7,9).

This complex process suggests that the SRP has multiple functions which may be reflected in the structure of the particle. A detailed understanding

of this structure may allow the identification of regions responsible for different steps of the process.

As a first approach to the study of the SRP function we have cleaved the RNA moiety of the ribonucleoprotein, using micrococcal nuclease to produce discrete subparticles which can be separately analyzed.

The results show that under the conditions used the RNA molecule within the SRP is highly resistant to nuclease digestion and the few nicks introduced generate discrete RNA fragments which are associated with different polypeptides. The 7SL RNA sequence may be described as a central S sequence inserted about two thirds from the 5' end of an Alu monomer sequence (1). The central S fragment of the 7SL RNA is bound to the four largest polypeptides while the 5' and 3' Alu ends of the molecule are bound to the 14 and 9 K proteins. Furthermore the introduction of a single nick between the 5' flanking sequence and the S fragment of the 7SL RNA causes no loss of function and no disruption of the particle.

MATERIALS AND METHODS

In vitro translation, membrane translocation and processing of nascent polypeptides, gel electrophoresis of proteins and isolation of rough microsomes from dog pancreas were previously described (5,10).

SRP purification and digestion with micrococcal nuclease

SRP isolated from the rough microsomes of dog pancreas by washing in 0.5 M KCl was purified by chromatography on ω-aminopentyl agarose followed by sucrose density gradient centrifugation (5,11,2). The digestion with micrococcal nuclease (Staphylococcus aureus nuclease, Boehringer) was carried out in a buffer containing 20 mM HEPES, pH 7.5, 2 mM $Mg(Ac)_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol and KAc 120-500 mM, at the temperature and for the time indicated. At the end of the incubation the nuclease was inactivated by addition of EGTA to 2 mM.

RNA extraction and fractionation

RNA was extracted from the SRP with phenol-chloroform and ethanol precipitated. Polyacrylamide-urea gels were according to Maniatis et al. (12). Polyacrylamide gel electrophoresis was according to Maniatis et al. (13).

The RNA fragments used for sequencing were obtained by digestion of the nuclease treated SRP 30 min at 37°C with 1 mg/ml proteinase K (Merck) followed by three phenol-chloroform, three chloroform extractions and alcohol precipitation.

The RNA was dephosphorylated using calf intestine alkaline phosphatase (Boehringer) and purified as described above. Labelling of the 3' end was carried out ligating overnight ^{32}P-pCp (Amersham 3000 Ci/mmole) to the RNA with T_4 RNA ligase (PL biochemicals) (14). The labelled fragments were separated in a 6% polyacrylamide-urea gel, identified by autoradiography and eluted. In order to obtain fragments with unique 3' end, we rerun them in a 10% sequencing gel (15) 2 mm thick. This was not necessary for fragments terminating with the natural 3' end of the 7SL RNA (fragments 1 and 5). The purity of each fragment was tested by complete digestion of the eluted RNA with an excess of RNAase T1 (PL biochemicals), followed by electrophoresis in a 20% sequencing gel. Only the RNA fragments giving one major digestion product were used for sequencing. In this way, we detected 2-3 different 3' ends for each fragment after cleavage with micrococcal nuclease (16).

RNA sequence analysis

Enzymatic sequencing of the RNA was carried out according to Donis-Keller et al. (15), with a kit from PL biochemicals and analysed on 10% and 20% sequencing gels. Mapping for adenine and guanine was sufficient to identify the fragments by comparison with the known 7SL RNA sequences (1,4).

RESULTS

Digestion of the SRP with micrococcal nuclease

Walter and Blobel (3) have shown that the digestion of the SRP with micrococcal nuclease inactivates the particle. We have analyzed the degradation products of the particle after micrococcal nuclease digestion and related the RNA damage to the loss of the SRP function.

Figure 1 shows the effect of micrococcal nuclease digestion of SRP at potassium acetate concentrations ranging from 0.12 - 0.5 M. Under all these conditions similar RNA fragments are produced, but the slower migrating component (band 1) is visible only at the higher salt concentrations. At lower salt only the faster migrating fragments (bands 2-4) can be detected. This suggests that the amount of enzyme necessary for the digestion is much lower in 0.12 M potassium acetate where probably the micrococcal nuclease is more active. However, earlier experiments showed that at lower salt, the SRP particle loses activity rapidly but is stable at higher potassium acetate concentrations (figure 1, lower inset). Subsequent experiments were done therefore at 0.4 M salt. Digestion under the same conditions of pure 7SL RNA produces complete degradation of the RNA (not shown). This suggests that bound polypeptides are responsible for the observed protection of the RNA.

Figure 2 shows that the cleavage of 7SL RNA occurs in steps. The first

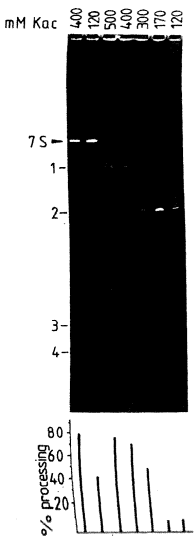

Figure 1. Digestion of the SRP at different salt concentration. SRP (1A_{280} unit/ml) was incubated with and without 1500 units/ml of micrococcal nuclease for 60' at 30°C, at the salt concentrations indicated above the slots. The first two samples on the left are without enzyme. An aliquot of each sample was extracted with phenol-chloroform, and the RNA was electrophoresed in a 6% polyacrylamide-urea gel and stained with ethidium bromide. The RNA bands are numbered from 1-4. The position of 7SL is indicated. An aliquot of each sample was added to an in vitro translation system to test the capacity to reconstitute translocation of the light chain of IgG across salt washed membranes (RM_K) (5). The products of translation were electrophoresed in a SDS-polyacrylamide gel (17). The densitometric analysis of the autoradiography of the gel is shown in the histogram (lower inset). The ordinate indicates the percentage conversion of pre-light into light chain. Under the same experimental conditions untreated SRP gives 80% processing (not shown). The alignment of the bars corresponds to the salt concentrations used for SRP digestion.

nick produces the RNA fragments 1 and 3 (lanes 2-8). Further degradation of the RNA occurs under stronger conditions of digestion (lanes 9 and 10). Under these conditions fragments 1 and 3 are further cut to produce fragments 2,4 and 5 (not visible in fig.2). The initial size of fragment 3 is also slightly reduced by stronger digestion. The observed pattern of degradation is highly reproducible. When enough RNA is electrophoresed or the RNA fragments are isotopically labelled, it is possible to detect minor RNA bands in addition to the major digestion products (fig.3a). This indicates the existence of rare cutting sites of the micrococcal nuclease in the SRP.

In order to relate the breakdown of the RNA to the function of the particle we have tested the capacity of the nuclease digested SRP to promote translocation of the light chain of IgG across membranes washed with salt (RM_K)(5). It is clear that after introduction of the first RNA cut the particle does not lose activity (Fig.2, lanes 2-8; fig.1, lower insets). However, stronger digestion of the SRP produces loss of function both at the level of translation and translocation (Fig.2, lanes 9,10 and fig.1).

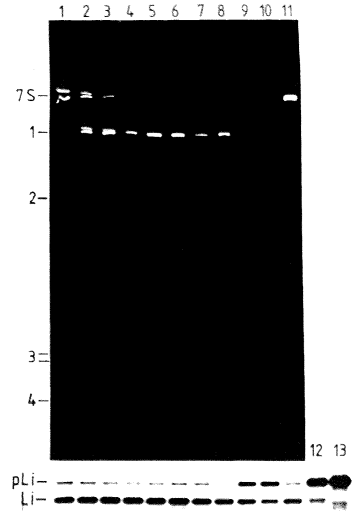

Figure 2. Analysis of nuclease digested SRP. SRP (1 A_{280} unit/ml) was incubated in 0.4 M KAc, with increasing concentration of micrococcal nuclease according to Materials and Methods. The RNA was extracted and electrophoresed as described in figure 1. 30 min incubation at 30°C with the following concentrations of enzyme, lanes: 1, minus nuclease; 2, 1000 units/ml; 3, 2000 units/ml; 4, 3000 units/ml; 5, 4000 units/ml; 6, 5000 units/ml; 7, 10000 units/ml. 60 min incubation at 30°C, lanes: 8, 5000 units/ml; 9, 10000 units/ml. 60 min incubation at 37°C: lanes 10 and 11, 10000 units/ml micrococcal nuclease. Before nuclease addition sample 11 was made 2 mM with EGTA, to block the activity of micrococcal nuclease. Aliquots of the nuclease digested samples 1-11 were assayed as described in figure 1. The autoradiograph of the protein gel is shown in the lower inset. pLi = prelight chain of IgG. Li = light chain of IgG. The slots of the protein and RNA gels are aligned according to the order of the samples. Protein synthesis in the presence and the absence of RM_K is shown respectively in slots 12 and 13.

Sequence analysis of the RNA fragments obtained from micrococcal nuclease digestion of the SRP

Walter and Blobel (3) have partially sequenced dog 7SL RNA and shown its extreme similarity to human and rat 7SL RNAs (1,4). The availability of such

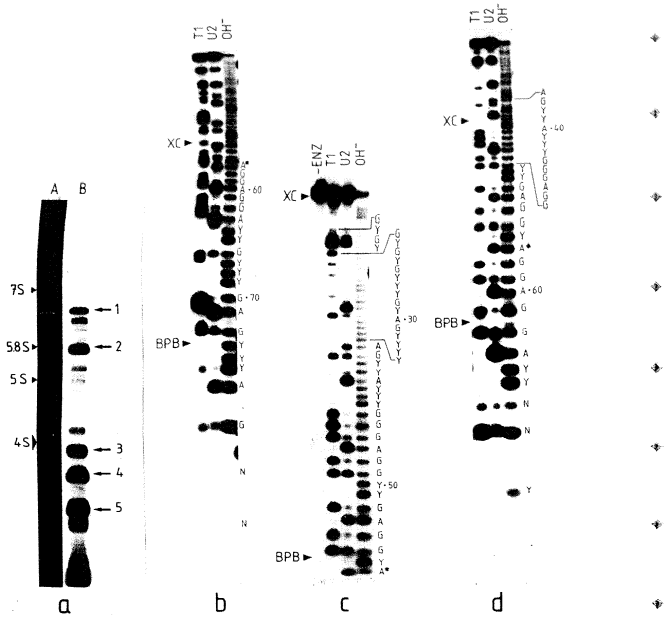

Figure 3. RNA sequence analysis of the 3' terminal portion of fragments 3 and 4. The labelled RNA was obtained from SRP (1 A_{280} unit/ml) digested 30 min. at 37°C with 10,000 units/ml micrococcal nuclease in 0.4 M KAc and electrophoresed in a 6% polyacrylamide-urea gel. a: ethidium bromide staining (A) and autoradiography (B) of the gel from which the RNA was eluted. b, c and d: sequencing gels of fragments: 3 (20% gel), 4 (10% gel) and 4 (20% gel). The corresponding nucleotides are shown on the side of the sequencing ladder and numbered according to the sequence of Ullu et al. (1). XC = xylene cyanole, BPB = bromophenol blue. T1 and U2 indicate the respective ribonucleases. OH^-: partial alkaline hydrolysis. -ENZ = untreated RNA. * identical to the rat in this position (4), but T-A transversion as compared with human 7SL RNA sequence.

sequences has simplified the identification of the RNA fragments obtained by micrococcal nuclease digestion of the SRP. RNA isolated from nuclease digested SRP was 3' end labelled and electrophoresed in a 6% acrylamide-urea gel, as described in Materials and Methods. Figure 3 shows the labelling pattern

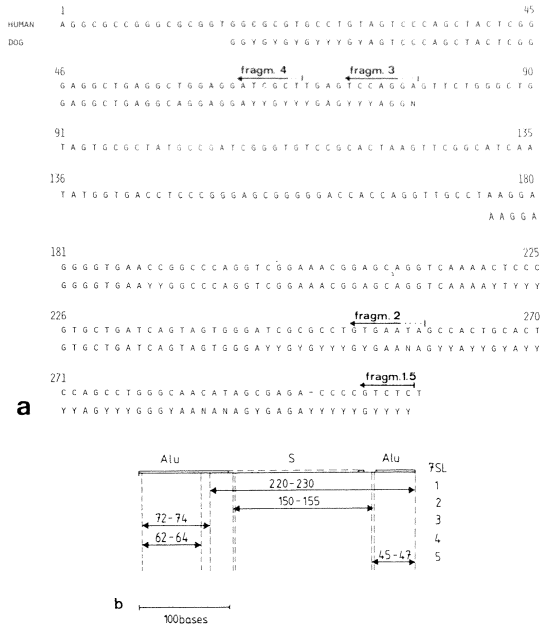

Figure 4. Alignment of the sequenced RNA fragments with the human 7SL RNA sequence. a: Y = pyrimidines. N = unidentified nucleotide. Where indicated, the sequence for all four bases was determined. The arrows indicate the 3' end of each fragment. The dotted line spans the region of cleavage of micrococcal nuclease. b: schematic representation of the dog RNA fragments, numbered from 1 to 5 according to size as in figure 3. The number of nucleotides for each fragment are given. The position of Alu and S sequences within the 7SL RNA are indicated above.

(lane B) compared with the ethidium bromide staining (lane A) of the RNA fragments. Although bands 4 and 5 are only barely visible in the film negative, the corresponding RNAs are efficiently labelled by ^{32}P-pCp. We decided to sequence these five RNA bands, which represent major products of digestion . We did not attempt to purify the very small RNA bands due to the large number of overlapping different RNA fragments present in this region. Since micrococcal

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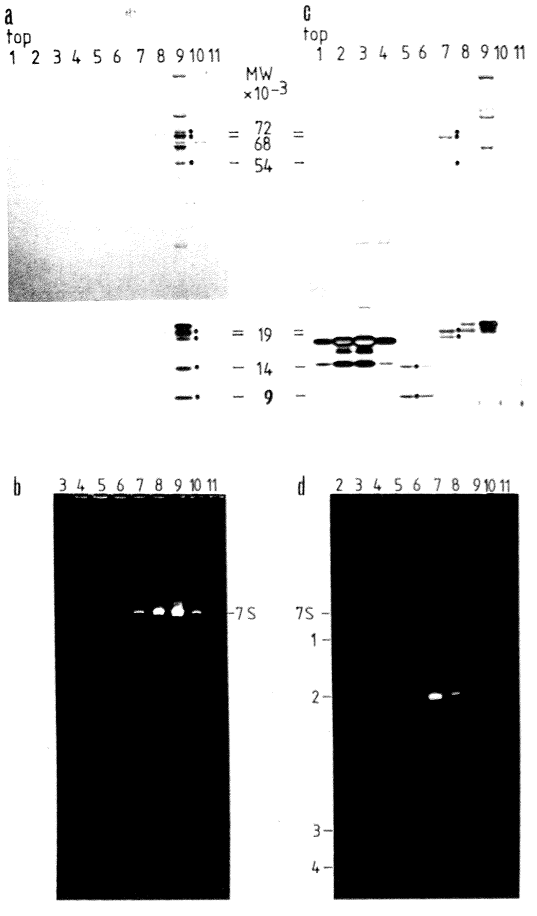

nuclease does not cut precisely and generates fragments different by a few bases (16) the RNA eluted from the gel of fig.3a was further electrophoresed in a gel which resolves RNA species differing by one base. Partial sequencing of the 3' end of the isolated fragments was sufficient to ascertain the position of each segment within the 7SL RNA sequence. Examples of sequencing gels are shown in fig.3b,c and d.

We determined the 5' end sequence of each RNA segment on the basis of its length. The number of bases was determined by electrophoretic analysis of the RNAs in a sequencing gel. The alignment of the fragments with the sequence of human 7SL RNA is shown in fig.4a. The scheme of fig.4b indicates the position and the length of the fragments resulting from nuclease digestion of the particle. The first cut, generating bands 1 and 3, occurs in a position corresponding approximately to base 80 of the human sequence. From the size of fragment 3 it seems that dog 7SL RNA has a 5' end similar to that of rat and shorter than that of the human sequence (1,4). Fragment 3 contains almost the entire 5' Alu flanking sequence of the molecule. Stronger digestion results in the trimming of fragment 3 which is shortened by approximately 10 bases at the 3' end, with the production of fragment 4. Furthermore, fragment 1 is cut in a position corresponding to approximately base 260 of the human sequence and shortened by approximately 25 bases at the 5' end. Thus fragments 2 and 5 are generated: one representing almost exclusively the S region and the other most of the Alu end of the 7SL RNA (1). The experiments show that all the RNA fragments derived from nuclease digestion of the SRP can be fitted together to reconstitute almost the entire sequence of the 7SL RNA.

Integrity of the SRP

In order to examine the effect of RNA degradation on the integrity of the SRP we have analysed the sedimentation properties of the products of nuclease digestion. Native SRP centrifuged in a sucrose density gradient migrates in a

Figure 5. Analysis of RNA and proteins of SRP separated on a sucrose density gradient. Micrococcal nuclease digested (c and d) and not digested (a and b) SRPs were centrifuged in a 5-20% sucrose density gradient containing 0.4M KAc, 20 mM HEPES pH 7.5, 1 mM dithiothreitol for 15 h at 45000 RPM, 4°C in a Spinco SW 60 rotor. The digestion was carried out in 0.4 M KAc for 60 min at 37°C with 10000 units/ml micrococcal nuclease. Proteins were purified from an aliquot of each gradient fraction and analysed in a SDS-polyacrylamide gel (a and c). The proteins were stained with Coomassie blue and subsequently the lower portion of the gel with silver, in order to visualize the smaller polypeptides (lower panels of a and c). The dots indicate the bands corresponding to the SRP proteins. The 19 K protein is present in two forms. The RNA was extracted from another aliquot and analysed as described in figure 1 (b and d). The numbers above the slots indicate the corresponding gradient fractions.

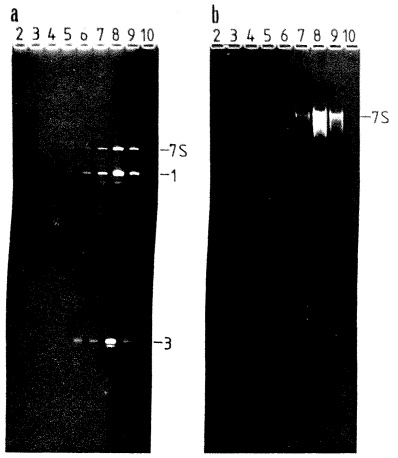

Figure 6. Denaturing and non-denaturing gels of RNA from nuclease treated SRP. SRP digested for 30 min at 30°C with 2000 units/ml micrococcal nuclease was centrifuged in a sucrose density gradient and fractionated, as described in figure 5. Aliquots of the RNA extracted from each fraction were electrophoresed in a 6% polyacrylamide-urea gel (a) and in a 10% polyacrylamide gel (b).

uniform peak in the approximate position of 11S. The analysis of the proteins and RNA of each gradient fraction shows the same composition throughout the peak (figure 5a and b, fractions 8 and 9). Centrifugation of SRP digested with 2000 units/ml micrococcal nuclease gives essentially the same result (figure 6a). Although the RNA is cleaved, the SRP migrates in the same position of the gradient as the intact particle (fractions 8 and 9) suggesting that RNA and proteins are still part of the same complex.

A different picture is observed when SRP digested with 10000 units/ml micrococcal nuclease is centrifuged (figure 5c and d). The sedimentation profile is broader than in the control experiment and at least two separate components can be visualized. One sedimenting faster and containing the RNA fragment 2 with the 72, 68, 54 and 19 K proteins (fraction 7). The second, slower sedimenting, contains fragments 3, 4 and 5 together with the 14 and 9 K proteins (fractions 5 and 6). Although it is clear that proteins are protecting these smaller RNA fragments, we cannot deduce which proteins are associated to which fragment and whether we are dealing with one or more subparticles.

Thus multiple nicks of the RNA seem necessary to disrupt the SRP. The first cleavage which separates the 5' end Alu fragment from the rest of the molecule does not seem to change the structure of the SRP. Under these conditions the particle may be held together by protein - RNA interaction or hydrogen bonding between the 5' end and other parts of the 7SL RNA or both. If hydrogen bonding is responsible for such a result, electrophoresis in a non-denaturing gel of the 7SL RNA from the digested particle should show a single band migrating in the position of 7SL RNA, instead of the two bands normally found in polyacrylamide-urea gels. The experiment of figure 6b shows that this is the case. After micrococcal nuclease digestion of the SRP, the extracted RNA gives only one visible band on polyacrylamide gel which corresponds to the position of 7SL RNA. After stronger digestion, the RNA pattern in a non-denaturing gel shows several bands smaller than 7SL RNA indicating that the fragments are no longer all held together (not shown).

DISCUSSION

A striking feature of the SRP is its very high resistance to micrococcal nuclease digestion. Since this enzyme digests readily free nucleic acids single or double stranded (18), the protection of the RNA molecule within the SRP must be due to the polypeptides which are shielding the RNA and limiting the access of the nuclease onto the molecule.

The cleavage of the molecule which occurs in subsequent steps suggests the existence of more and less accessible sites. The first nick at the 5' end does not alter the function nor the integrity of the SRP, suggesting that the two RNA fragments may be held in place by the presence of protein(s) and, or, hydrogen bonding. The possibility of base pairing within the RNA molecule is shown by the experiment of figure 6. In agreement with this observation, inspection of the sequence shows that the 5' and 3' Alu flanking sequences of 7SL RNA could base pair with each other forming a stable duplex.

The introduction of several nicks in the RNA is concomitant with the disruption of the particle and results in the loss of SRP function. We do not know whether it is the RNA damage or the loss of integrity of the SRP or both that result in a loss of function.

The inactive subparticles obtained from nuclease digestion give some indication of the distribution of the proteins on the RNA chain. Four poly-

peptides are bound to the 150 nucleotides fragment while the 14 and 9 K proteins comigrate with the smaller fragments, indicating a fairly even distribution of the proteins along the RNA molecule. However we do not know whether all the proteins are bound to the RNA or if protein-protein interaction also plays a role in the structure of the SRP.

The products of cleavage of the SRP reflect very closely the bipartite structure shown by the sequence analysis of the RNA. Ullu et al. (19) have shown that the S sequence of 7SL RNA is highly conserved in evolution, while the Alu sequence is not. We propose that the S fragment bound to four polypeptides may represent a structural domain of a more primitive particle. The insertion of the S sequence into an Alu monomer may have generated a second domain. A more detailed functional analysis of the particle may answer this question.

ACKNOWLEDGEMENTS

We are grateful to V. Pirrotta for reading the manuscript and to Ms W. Moses for typing it. E.D.G. was the recipient of a long term EMBO fellowship (ALTF 207-1982).

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Volume 11 Number 21 1983 Nucleic Acids Research

Isolation and translation of mRNA coding for the variant surface antigens of *Paramecium*

John Sommerville

Department of Zoology, University of St. Andrews, St. Andrews, Fife KY16 9TS, UK

Received 25 August 1983; Revised and Accepted 14 October 1983

ABSTRACT

In the poly(A)$^+$RNA isolated from the ciliate Paramecium primaurelia is found a discrete and abundant mRNA species of high molecular weight (corresponding to about 9,000 nucleotides). This mRNA species has size and abundance characteristics that identify it tentatively as the message coding for the variant cell-surface antigens. After microinjection of the high molecular weight mRNA into amphibian oocytes, polypeptides are synthesized that are immunoprecipitated specifically with antibodies directed against the homologous Paramecium antigen. On collecting the culture medium of oocytes microinjected with Paramecium mRNA, newly-synthesized complete antigen molecules ($M_r \sim 300,000$) can be recovered by immunoprecipitation.

INTRODUCTION

The cell-surface antigens of Paramecium are in several ways remarkable proteins: each consists of an extremely large ($M_r \sim 300,000$) single polypeptide /1,2/; about 10% of the amino acid residues are cysteine, all of which appear to be involved in disulphide linkage, generating about 125 such bonds per polypeptide /3,4/; the expressed antigen is secreted to the cell surface where it forms a complete covering of the cortex and cilia and accounts for 3.5% of the cell protein /5/. A complex regulatory mechanism exists whereby only one of the family of genes coding for different surface antigens is expressed at any one time /6/.

In P. primaurelia, antigen (serotype) expression is influenced by temperature. The effect of temperature, and other environmental stimuli is through cytoplasmic components rather than on the genes directly /7/. By manipulating the temperature of cell culture, the expression of antigen can be made to switch from one type to another in a controlled manner. In stock 168, the 24°C to 32°C induced switch from serotype G to D is accompanied by changes in the pattern of mRNA production /8/.

Evidence has been presented for both transcriptional /8,9/ and translational /10/ regulation of antigen-coding gene activity. Such investigations