Volume 13 Number 2 1985 Nucleic Acids Research

Complexes of viroids with histones and other proteins

Petra Wolff, Ruth Gilz, Jürgen Schumacher and Detlev Riesner

Institut für Physikalische Biologie, Universität Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf 1, FRG

Received 22 November 1984; Revised and Accepted 3 January 1985

ABSTRACT

Complexes of potato spindle tuber viroid (PSTV) with nuclear proteins have been studied by in vitro reconstitution of the complexes and by isolation and characterization of in vivo complexes under non-dissociating conditions. For in vitro reconstitution, nuclear proteins were separated by SDS-gelelectrophoresis, renatured and blotted onto nitrocellulose filters, and incubated with viroid. The viroid-protein complexes were crosslinked covalently, and the viroid containing protein bands were detected by northern hybridization with a radioactive cDNA probe. The histones, a 41,000 dalton protein and to a small extent a 31,000 dalton protein were found in complexes with viroids. Raising the strength to 0.4 M NaCl destroys the complexes with the 41,000 dalton proteins but not those with the histones.

From nucleoli, which are known to obtain the majority of viroids under non-dissociating conditions (Schumacher et al., (1983) EMBO J. 2, 1549-1555), a nucleosomal fraction was prepared. Viroids were found predominantly in this nucleosomal fraction. They are bound in a complex of 12-15 svedberg units.

INTRODUCTION

Viroids are the causative agent of several diseases of higher plants. They are single-stranded, circular RNAs composed of 240 - 370 nucleotides; they are distinguished from viruses by the lack of a protein coat and by the small size of their genome (reviewed in 1 - 4). Viroids follow a unique principle of structure and dynamics (5). They adopt a rodlike secondary structure, formed by an unbranched arrangement of regions of intramolecular basepairing alternating with loops. The thermal denaturation of viroids is highly cooperative and runs through intermediate hairpin structures, which are not present in the native viroid (6).

Although the structure of isolated viroids is known in great detail, the problem of the in vivo structure of viroids has been approached only recently. It was found that nearly the whole PSTV-RNA is present in the cell nucleus; from further subfractionation it was concluded that viroids are associated with the nucleoli (7). These complexes can be abolished by raising the ionic strengh of the buffer to 0.66 M. It strongly indicates that viroids are com-

plexed in the nucleolus via a protein-nucleic acid interaction.

The aim of this paper is mainly to study the protein component of the cellular viroid-complexes. It is studied as well by reconstitution of the complexes in vitro as by characterization of the in vivo complexes. Viroid-binding proteins in vitro are identified by a combination of several methods: a western blotting of the proteins (8), an incubation and crosslinking with non radioactive viroids, and the detection of the viroids by hybridization with radioactive complementary DNA. The characterization of the viroid-protein-complexes in vivo is performed by standard procedures as differential centrifugation and sucrose gradients.

MATERIALS AND METHODS

Nucleic acids

Potato spindle tuber viroid (PSTV), grown from a strain originally supplied by T.O. Diener, Beltsville, Maryland, was purified from infected tomato tissue (Lycopersicon esculentum) as described (9). It was generously provided by Dr. M. Colpan (this laboratory).

Viroid cDNA used for molecular hybridization was obtained from the plasmid pAV 401, which is a derivative of pBR 322 with the viroid cDNA inserted into the Bam HI site (10). It was kindly provided by Prof. A.v. Kammen.

Enzymes

DNA-dependent RNA polymerase II from wheat germ (E.C. 2.7.7.6), prepared and stored as described (11,12) was a gift of L. Nagel and Dr. Th. Goodman (this laboratory). DNase I (E.C. 3.1.4.5), 11000 units/mg, was purchased from Worthington. Alkaline phosphatase (E.C. 3.1.3.1), 500 units/mg, was obtained from Serva. The suspension in 3 M $(NH_4)_2SO_4$, pH 7.0 was dialysed against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and adjusted to 0.02 units/µl. T4-polynucleotide-kinase (E.C. 2.7.1.78) was a product of Boehringer Mannheim.

Preparation of nuclei

Nuclei were prepared from PSTV-infected tomato leaves (Lycopersicon esculentum) by the method of Schumacher et al. (7). In contrast to that publication (page 1554), the nuclei were not suspended in buffer D as written erroneously due to a misprint, but in buffer E.

Isolation of subnuclear fractions

The components of the nuclei were fractionated according to Pederson (13). A chromatin-containing fraction and a nucleoli-containing fraction were harvested for further use.

Preparation of a nucleosomal fraction from nucleoli

The nucleoli-containing fraction was resuspended in 5 ml 10 mM Tris-HCl, pH 7.0, 10 mM NaCl, 15 mM $MgCl_2$ and incubated at 37°C with 1100 units DNase I for 2 h. The digested nucleoli were lysed by brief sonication (3 x 10 s, 45 W, Brown Labsonic 1510 with standard probe). The sonicate was layered on 25 ml 30 % (w/v) sucrose (RNase-free) in 0.02 M EDTA, pH 8.0 and centrifuged at 5000 r.p.m., 4°C for 15 min in an SW28 rotor (Beckman). Three fractions were harvested: the pellet, the sucrose cushion and the supernatant consisting of the layer on top of the cushion and the interphase. The fractions were analysed by gelelectrophoresis. The top fraction contained the nucleosomal fractions.

Preparation of nuclear proteins

Proteins from nuclei resp. from the subnuclear fractions were isolated according to Bloom and Anderson (14) by the hydroxylapatite dissociation method with minor modifications.

Gelelectrophoresis

SDS-polyacrylamide-electrophoresis (SDS: sodium dodecyl sulfate) of proteins was carried out as described by Laemmli (15). The proteins were made up 2% SDS, 5% 2-mercaptoethanol, 20% glycerol and heated to 80°C for 5 min before application to the gel. The gels were stained according to Oakley et al. (16).

For viroid detection the "bidirectional" gelelectrophoresis was used as published by Schumacher et al. (17). As little as 0.1 ng viroid-RNA may be detected by this analysis after applying the silver staining procedure of Sammons (18). All slab gels were 14 x 10 x 0.15 cm using a thermostated apparatus (Bio-Rad Model 220).

Western-blotting of nuclear proteins

The gel containing the proteins of nuclei after SDS-electrophoresis was immersed in 200 ml renaturation-buffer (50 mM NaCl, 2 mM EDTA, 0.1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.0) for 2 h. During this step most of the SDS was removed from the proteins and the gel, and the subsequent renaturation of the proteins was facilitated (8). The transfer of the proteins to nitrocellulose filters (BA 85, Schleicher und Schüll) was carried out as described by Southern (19). The transfer-buffer was 0.5 M NaCl, 2 mM EDTA, 0.1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.0; the duration of the transfer was about 72 h.

Binding of viroid-RNA to the blotted proteins

After protein-transfer the nitrocellulose filters were immersed in binding buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 x Denhardt's reagent (0.02% BSA (bovins serum albumin), 0.02% Ficoll, 0.02% polyvinylpyrrolidone)). The filters were placed into a plastic bag, 10 ml of binding buffer containing

20 ng/ml PSTV-RNA were added, and incubation to establish RNA binding was allowed for 60 min at room temperature (8). Background binding was reduced by washing the filter in 150 ml binding buffer for 45 min at room temperature.

Fixation of the RNA-protein-complex

The RNA-protein-complexes were fixed using the crosslinking reagent glutaraldehyde (20). The filters were incubated for 10 min at 30°C in 0.25% glutaraldehyde, 5 mM K-phosphate, pH 6.8.

Preparation of the hybridization probe

The E. coli strain HB 101 containing the plasmid pAV 401 was grown in M9 minimal medium with 25 µg/ml penicillin G in a 4.5 l fermentor that was built in the university workshop. Cell growth and isolation of plasmids were carried out as presented by Hillen et al.(21). The plasmid sample was further purified by a CsCl density centrifugation following standard procedures. After centrifugation the pellet was suspended in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA to a final concentration of about 1 mg/ml. PSTV-cDNA was excised from the plasmid by BAM HI digestion. The enzyme was removed by 3 extractions with an equal volume of phenol and traces of the latter by 3 extractions with ether, and the DNA was precipitated with 3 vol. of ethanol. cDNA and vector DNA were separated by HPLC using Nucleogen-DMA 4000 (22). The isolated cDNA was resuspended in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

Hybridization of viroids

The isolated cDNA was labeled at the 5'end with γ^{32}P-ATP according to the method of Chaconas and van de Sande (23). The volume was reduced to 5 µl to decrease the amount of γ^{32}P-ATP.

The hybridization procedure followed the method of Thomas (24) with some modifications: the filters were sealed in commercial plastic bags and prehybridized in 50% deionized formamide, 50 mM Na-phosphate, pH 6.5, 5 x SSC (1 x SSC: 150 mM NaCl, 15 mM Na-citrate, pH 7.0), 1 x Denhardt's reagent, 250 µg/ml denatured salmon sperm DNA for 4-16 h at 42°C. For hybridization 4 parts of fresh prehybridization buffer were mixed with 1 part of 50% (w/v) dextransulfate; the mixture was degassed to decrease the viscosity and adjusted to 2 ng/ml 5'-endlabeled cDNA. Hybridization was performed at 65°C for at least 4 h. The filters were washed 3 times with 2 x SSC, 0.1% SDS at room temperature for 5 min each and 3 times with 0.1 x SSC, 0.1% SDS at 60°C for 15 min. These conditions are highly specific for cDNA-PSTV hybridization. The damp filters were again sealed in plastic bags and exposed to a Kodak X-omat S film using DuPont Cronex Lightning-Plus intensifying screens.

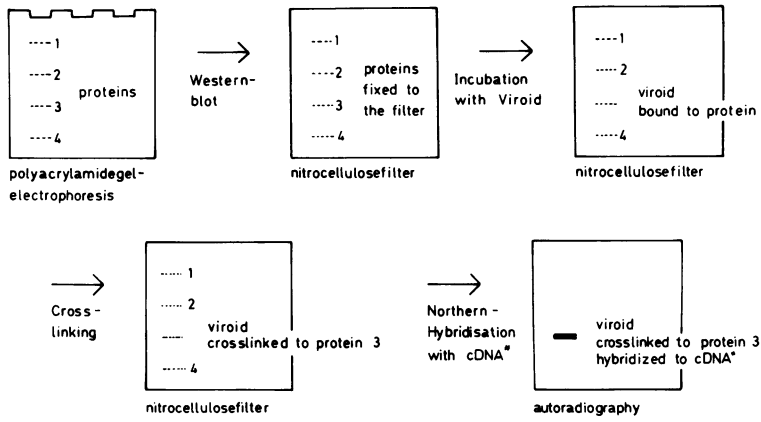

Fig. 1: Procedure to detect in vitro reconstituted PSTV-protein-complexes. Step 1: separation of the proteins by SDS-gelelectrophoresis; step 2: transfer of the proteins to nitrocellulose-filters (western blot); step 3: incubation with viroid-RNA to establish the binding; step 4: fixation of the complexes by crosslinking with glutaraldehyde; step 5: detection of the viroid-RNA by molecular hybridization using ^{32}P-endlabeled cDNA (nothern hybridization).

RESULTS

In vitro reconstitution of protein-PSTV-complexes

In order to search for viroid binding proteins, the proteins of the nucleus were fractionated and the various fractions tested for viroid binding. It had to be tested whether viroid-protein-complexes identified by the in vitro reconstitution are of any significance.

Specificity of the method

A summary of the procedure used to detect viroid-protein-complexes is shown in Fig. 1. The method is based on the reconstitution of non-covalently bound complexes. These are covalently fixed in a second step, because they would dissociate otherwise during the subsequent detection of the viroid-RNA by molecular hybridization.

The specificity of the method in detecting viroid-protein-complexes was proven by using the system PSTV and DNA-dependent RNA polymerase II from wheat germ. The equilibrium constant for the viroid-polymerase interaction is $1.9 \times 10^7\ M^{-1}$ (25), which is in a physiologically reasonable range. Dot-blots were used in these experiments, because a pure fraction of polymerase was available. The results of these control experiments are shown in Fig. 2.

The first control (lane 2) shows that viroid RNA is necessary to detect the cDNA bound to the complexes. If, however, the filter is incubated with buffer

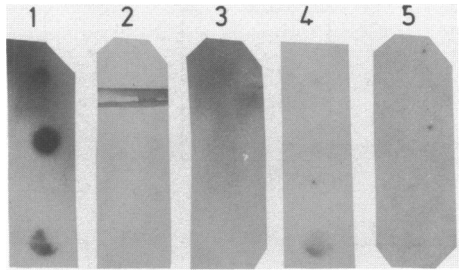

Fig. 2: In vitro reconstitution of viroid-protein complexes as tested with polymerase II. In each lane three different polymerase II concentrations were used: 0.5 µg, 2.5 µg, and 5 µg enzyme from top to bottom. Lane 1: procedure as described in fig. 1, but dot blots are used instead of transferring the proteins from the gel onto nitrocellulose-filters, lane 2 - 5: controls with the following differences to the standard procedure; lane 2: incubation with buffer instead of PSTV-RNA; lane 3: incubation with buffer instead of PSTV RNA, no fixation with glutaraldehyde; lane 4: no fixation with glutaraldehyde; lane 5: BSA, cytochrom c, ferritin (5.0 µg each) are blotted onto the filters instead of polymerase.

instead of PSTV-RNA, and treated with glutaraldehyde, no radioactivity is detectable on the filters. Thus binding of the labeled cDNA can be excluded. The second control (lane 3) was performed in absence of viroid-RNA as well as glutaraldehyde fixation. No radioactivity could be detected on the filters showing that in these experiments the cDNA does not form a detectable complex with polymerase, if it is not mediated by crosslinked viroid-polymerase complexes. Last the effect of glutaraldehyde fixation is tested by performing the procedure omitting the glutaraldehyde fixation step (lane 4). Only the 20-fold concentration of enzyme in comparison to the complete procedure (lane 1) shows a radioactive signal, i.e. viroid-protein-complexes. The last dot was dispersed unintentionally during application and therefore shows less intensity. The fixation step enables the detection of viroid-binding using low concentrated probes. If not crosslinked, most of the RNA bound to the proteins dissociates during the hybridization procedure. To demonstrate that no unsignificant complexes are formed, BSA, cytochrom c and ferritin were blotted onto nitrocellulose filters and the complete procedure was performed again (lane 5). No signals of bound viroid were found in the autoradiograph.

Reconstitution of viroid-complexes with nuclear proteins

Nuclear proteins were prepared from $3 \cdot 10^8$ nuclei, which is the yield from 150 g leaf material from tomato. 0.25% of the protein preparation obtained either from nuclei, chromatin- or nucleoli-containing fractions resp. were analysed by SDS-polyacrylamide gelelectrophoresis and transferred to nitro-

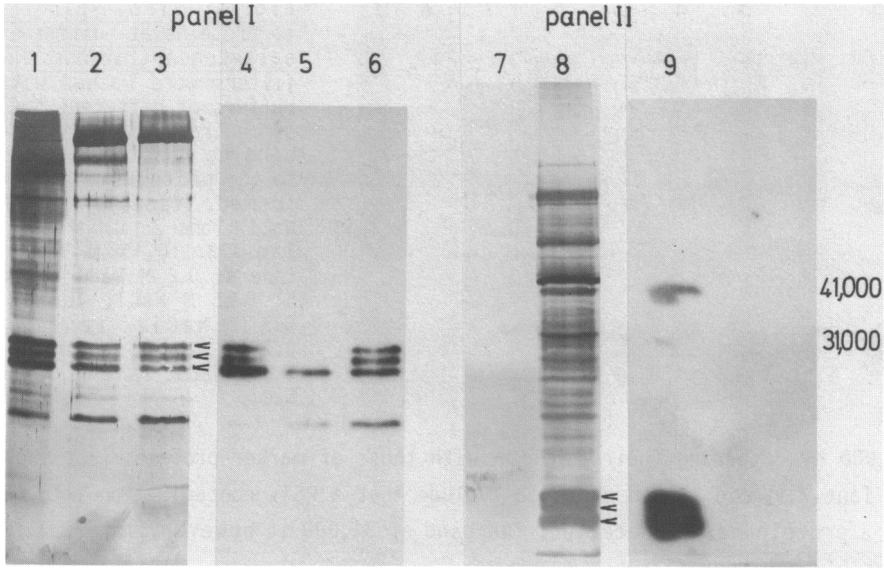

Fig. 3: In vitro viroid-protein complexes. Panel I, lane 1-3: silver-stained SDS-polyacrylamide gel, lane 4-6: detection of viroid-binding by autoradiography after electrophoresis under identical conditions as in lane 1-3; lane 1+4: proteins from cell nuclei; lane 2+5: proteins from chromatin; lane 3+6: proteins from nucleoli. Panel II: lane 7: viroid-RNA in a stained SDS-protein-gel; lane 8: proteins from nuclei in a stained SDS-gel; lane 9: detection of viroid-binding proteins from nuclei by autoradiography after long exposure. The gel runs in Panel I and II are of different length; the arrows point to the main histone bands.

cellulose filters. It was necessary to use denaturating gels, because the resolution of native protein gels was very poor.

Fig. 3, panel I shows the staining pattern of the proteins from the nuclei in lane 1, from the chromatin in lane 2 and from nucleoli in lane 3. The autoradiograph identifies those proteins of the three fractions (lane 4, 5, 6), which bind viroid-RNA at a salt concentration of 0.05 M NaCl. Comparing the band pattern on the autoradiograph with that on the stained gel, several viroid binding proteins were detected. They were identified as histones by their position in the gel and their relative concentrations.

A second experiment differed from that described above by changing the duration of the western blot from 72 h to 86 h. The result is shown in Fig. 3, panel II. Comparing the autoradiograph (lane 9) and the stained SDS gel (lane 8) again the histone-bands, and in addition two other viroid binding proteins were detected. Their molecular weights were estimated as 41,000 and

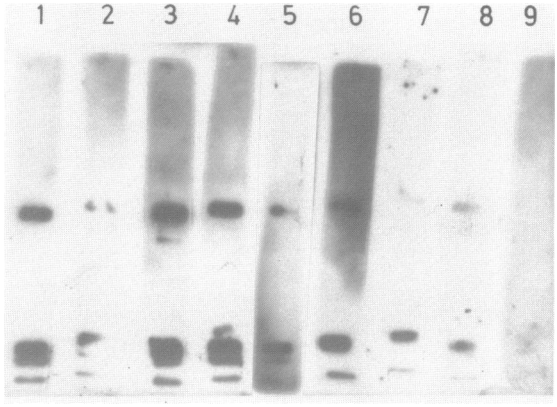

Fig. 4: Viroid-binding to proteins at different salt-concentrations: The filters were washed with buffers of different NaCl concentrations after the binding reaction of PSTV to the proteins was performed. lane 1: 0.05 M NaCl; lane 2: 0.1 M NaCl; lane 3: 0.15 M NaCl; lane 4: 0.2 M NaCl; lane 5: 0.25 M NaCl; lane 6: 0.3 M NaCl; lane 7: 0.35 M NaCl; lane 8: 0.4 M NaCl; lane 9: 0.5 M NaCl.

31,000 by comparing their position with those of marker proteins (not shown). In lane 7 viroid RNA is shown to exclude that a PSTV contamination not bound to a protein may be detected. The band of 31,000 is however, very weak and possibly not significant.

In order to obtain information about the strength of the complexes the filters were washed with binding buffers containing 0.1 to 0.5 M NaCl, after the binding reaction but before the crosslinking reaction had been carried out. As shown in Fig. 4 washing with 0.4 M NaCl dissociates the complex of viroid with the 41,000 dalton protein; the histone-viroid-complexes, however, cannot be totally dissociated even with 0.5 M NaCl. The intensity of the band of the 41,000 dalton protein is definitely higher in Fig. 4 compared to Fig. 3. This is due to the longer exposure of the autoradiogram. The variation of the relative intensities of the band of the 41,000 dalton protein and the histones comes probably from minor variations in the experimental conditions and from nonlinearities in the photographic densities.

In vivo complexes of viroids

After establishing viroid-protein complexes in vitro, the main emphasis was directed to the problem whether these or similar complexes may be prepared from the nucleus without using dissociating conditions.

Nucleosomal fractions of nucleoli

From 150 g leaf material nucleoli were prepared, and subsequently the nucleoli were fractionated into a supernatant fraction, the sucrose cushion and a pellet as described under Methods. According to the literature the supernatant contains the nucleosomes.

The nucleosomes obtained by this procedure are incomplete because the digestion with DNase I yields intranucleosomal cuts (26). In this experiment

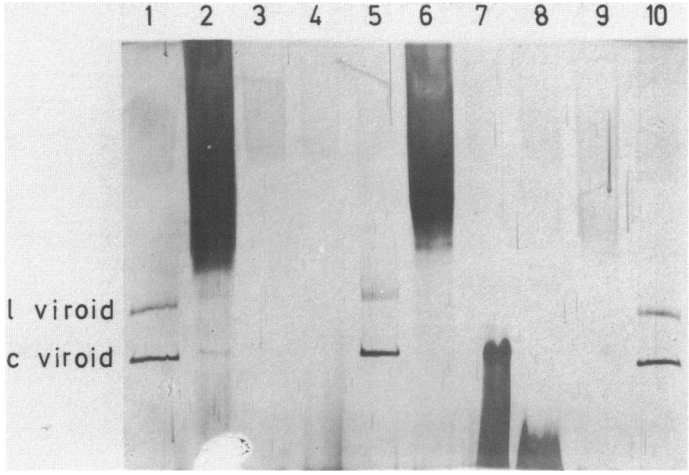

Fig. 5: Viroid detection in nucleosomal fractions by bidirectional gelelectrophoresis. Lane 1: viroid RNA as reference (the lower band is the circular viroid (c) and the upper band the linear form (l)); lane 2: supernatant from nucleoli-digest; lane 3: sucrose cushion from nucleoli-digest; lane 4: pellet from nucleoli-digest; lane 5: viroid RNA; lane 6: supernatant from chromatin-digest; lane 7: interphase from chromatin-digest; lane 8: sucrose cushion from chromatin-digest; lane 9: pellet from chromatin-digest; lane 10: viroid RNA.

micrococcal nuclease could not be used, because viroid RNA would be digested. As a control experiment nucleosomes were also prepared from the chromatin containing fraction applying the same procedure. All fractions (supernatant, cushion and pellet) obtained from nucleoli and slot with interphase from chromatin were tested for the presence of viroid by bidirectional gelelectrophoresis. The result is shown in Fig. 5.

Only in the nucleosomal fraction from nucleoli viroid RNA could be detected. The gelelectrophoretic analysis of the proteins present in that fraction showed the histones in the ratio which is known for nucleosomes, and small amounts of other proteins (data not shown). The centrifugation of the chromatin digest resulted a precipitate in the interphase which was analysed in Fig. 5, lane 7. The smear in the lower part of the slot is most probably not nucleic acid but protein. It is definitely no viroid because of the complete absence of the linear form.

Characterization of viroid containing complexes

The viroid containing complexes from the nucleosomal fraction were further characterized by their sedimentation behaviour. The nucleosomal fraction was layered on a 30 ml sucrose gradient, 5% - 25% (w/v) in 0.02 M EDTA, pH 8.0 and

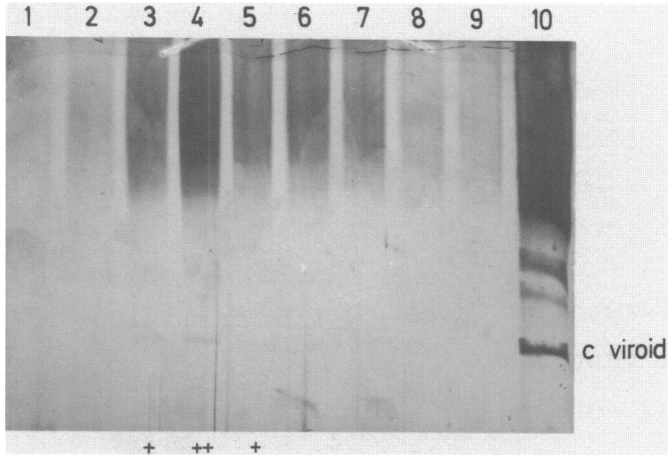

Fig. 6: Viroid-detection in a sucrose gradient of the nucleosomal fraction from nucleoli by bidirectional gelelectrophoresis. The nine slowest fractions (starting from the top) were tested for the presence of viroids. Lane 1 - 9: fraction 1 - 9; lane 10: PSTV containing crude extract as reference. The viroid bands in lane 3-5 are weak but clearly visible; the crosses indicate their relative intensities in the original gel.

centrifuged at 22,000 r.p.m., 4°C for 22 h in an SW28 rotor (Beckman). Fractions of 2.5 ml were collected and tested for the presence of viroids. Fig. 6 shows the gelelectrophoretic analysis from the 9 top fractions harvested from the gradient.

PSTV RNA was detected in the fractions 3, 4, 5. The position of the viroid RNA in the gradient allows the evaluation of rough $S_{20,w}$-values of the viroid containing complexes (27). The three fractions of the gradient containing viroid RNA correspond to the following $S_{20,w}$-values: 7.5 S, 12 S and 15 S.

DISCUSSION

In vitro reconstitution by combined western blotting and northern hybridization

In order to identify viroid binding proteins by *in vitro* reconstitution, nuclear proteins were separated by SDS-gelelectrophoresis and tested for viroid binding in the following way: the proteins were transferred to nitrocellulose filters by western blotting, non-covalent viroid-protein complexes were formed in an incubation step, the complexes were covalently fixed by cross-linking, and the viroid RNA detected in the complex by northern hybridization with a radioactive cDNA probe.

In comparison to other procedures detecting protein-nucleic acid complexes, affinity chromatography (28) or sucrose gradient and filter binding (29,30) e.g., the method used in this work has several advantages: using gelelectrophoresis to separate the proteins, a high resolution of the different proteins was obtained, which would be further improved by the application of two dimensional gels (31). Only very small amounts of proteins and nucleic acids were necessary for the experiments described (about 1 µg protein, 1.5 µg DNA, 0.2 µg PSTV-RNA per experiment). Furthermore, the procedure is easy to perform.

Problems arise, however, when the proteins are separated under denaturing conditions as in SDS before blotting. Proteins dissociate into their subunits and may not be able to bind viroid-RNA, because only the complete or a core-protein may show binding activity. The second effect of the SDS is the destruction of the secondary structure of the protein chains. Therefore, after electrophoresis a renaturation step is performed, where most of the proteins renature (8).

The procedure was tested by binding viroid-RNA to DNA-dependent RNA polymerase II. This control experiment yielded additional information about the general applicability of the procedure. Eucaryotic polymerases consist of at least 13 subunits (13), i.e. they are very complex. Even these complex enzymes show binding of nucleic acids after being adsorbed on nitrocellulose filters. Obviously, the fixation on the filter does not impair the binding activity of enzymes when transferred to the filter in their native conformation.

Viroid-binding proteins

In in vitro reconstitution experiments several viroid binding proteins have been detected: histones, a 41,000 dalton protein, and a 31,000 dalton protein in a weak band.

The complexes of viroids with the 41,000 dalton protein have been established only in in vitro experiments. Therefore, a physiological significance of these complexes cannot be inferred at present. It is , however, remarkable that among the many nuclear proteins (cf. Fig. 3) besides the histones only one or probably two proteins show a high affinity to viroids. It is hard to believe that this affinity is purely accidental.

The binding of viroids to histones is in accordance with the idea of the DNA-similarity of viroids. DNA-similarity of viroid was argued first on the basis of their structural and thermodynamic properties (6). Furthermore, it was found that viroid is accepted as a template by the DNA-dependent RNA polymerase II (25, 32). Earlier studies had already suggested an interaction

between viroids and histones. The experiments were performed, however, with histones from chicken erythrocytes, and the viroid-histone complexes were inferred only in an indirect manner (33). Histones bind to viroid independent whether the histones were prepared from chromatin or nucleoli. This result is not in contradiction to the location of viroids in nucleoli but is a consequence of the in vitro reconstitution of the complexes.

Still the viroid-histone complexes may be completely unspecific, lacking of any physiological relevance and may reflect only the basic character of the histones. Therefore it was important to characterize the in vivo complexes of viroids. Indeed, also in vivo viroids were found together with the histones of the nucleoli. Preparing the nucleosomal fraction from nucleoli, viroids were found in this fraction. Moreover, the sedimentation experiment showed that in this fraction the majority of viroids were not present as free molecules but bound in complexes of S-values between 12 and 15S. These S-values are characteristic for nucleosomes and oligomers of nucleosomes (34).

The results of this work clearly confirm the earlier finding of location of mature viroids in nucleoli. Going beyond the earlier results, the trace of mature viroids may now be followed into that part of the nucleoli, in which histones and ribosomal DNA form nucleosomal structures.

ACKNOWLEDGEMENTS

We like to thank Prof. A. van Kammen for providing the clone HB101, Dr. M Colpan for the gift of PSTV and Dr. Th. Goodman and L. Nagel for the gift of polymerase II. The work was supported by the Deutsche Forschunsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. Sänger, H.L. (1982)in: Encyclopedia of Plant Physiology, New Series Parthier, Boulter, D.,Eds.) Vol. 14B, pp.368-454, Springer Verlag, Berlin.
2. Diener, T.O. (1984) Trends Biochem. Sci. 9, 133-136.
3. Gross, H.J. and Riesner, D. (1980) Angew. Chem. Int. Ed. Engl. 19, 231-243; Angew. Chem. German. 92, 23-245.
4. Diener, T.O. (1979) "Viroids and Viroid Diseases", John Wiley and Sons, New York.
5. Riesner, D., Steger, G., Schumacher, J., Gross, H.J., Randles, J.W., and Sänger, H.L. (1983) Biophys. Struct. Mech. 9, 145-170.
6. Riesner, D., Henco, K., Rokohl, U., Klotz, G., Kleinschmidt, A.K., Domdey, H., Jank, P., Gross, H.J., and Sänger, H.L. (1979) J. Mol. Biol. 133, 85-115.
7. Schumacher, J., Sänger, H.L., and Riesner, D. (1983) EMBO J. 2, 1549-1555.
8. Bowen, B., Steinberg, J., Laemmli, U.K., and Weintraub, H. (1980) Nucl. Acids Res. 8, 1-20.
9. Colpan, M.,Schumacher, J., Brüggemann, W., Sänger, H.L., and Riesner, D. (1983) Anal. Biochem. 131, 257-265.

10. van Wezenbeek, P., Vos, P., van Boom, J., and van Kammen, A. (1982) Nucl. Acids Res. 10, 7947-7957.
11. Jendrisak, J.J. and Burgess, R.R. (1975) Biochemistry 14, 4639-4645.
12. Jendrisak, J.J. and Burgess, R.R. (1977) Biochemistry 16, 1959-1964.
13. Pederson, T. (1970) Proc. Natl. Acad. Sci. USA 71, 617-621.
14. Bloom, K.S. and Anderson, J.N. (1977) J. Biol. Chem. 253, 4446-4450.
15. Laemmli, U.K. (1970) Nature 227, 680-685.
16. Oakley, B.R., Kirsch, D.R., and Morris, N.R. (1980) Anal. Biochem. 105, 361-363.
17. Schumacher, J., Randles, J.W., and Riesner, D. (1983) Anal.Biochem. 135, 288-295.
18. Sammons, D.W., Adams, L.D., and Nishizawa, E.E. (1981) Electrophoresis 2, 135-141.
19. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
20. Renz, M. (1983) EMBO J. 2, 817-822.
21. Hillen, W, Klein, R.D., and Wells, R.D. (1981) Biochemistry 20, 3748-3756.
22. Colpan, M. and Riesner, D. (1984) J. Chromatogr. 296, 339-353.
23. Chaconas, G. and van de Sande, J.H. (1980) Methods Enzymol. 65, 75-85.
24. Thomas, P. (1980) Proc. Natl. Acad. Sci. USA 77, 5201-5205.
25. Goodman, T.C., Nagel, L., Rappold, W., Klotz, G., and Riesner, D. (1984) Nucl. Acids Res. 12, 6231-6246.
26. Palen, T.E. and Cech, T.R. (1984) Cell 36, 933-942.
27. Griffith, O.M. (1979) Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation (ed. Fa. Beckman)
28. Alberts, B.M., Amido, F.J., Jenkins, M., Gutmann, E.D., and Ferris, F.L. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 289-305.
29. Hinke, D.C. and Chamberlain, M.J. (1972) J. Mol. Biol. 70, 157-185.
30. Riggs, A.D. and Bourgeois, S. (1958) J. Mol. Biol. 34, 361-374.
31. O'Farrell, H.P. (1975) J. Biol. Chem. 250, 4007-4021.
32. Rackwitz, H.R., Rohde, W., and Sänger, H.L. (1981) Nature 291, 297-301.
33. Henco, K. (1979) Thesis, Hannover.
34. Bode, J. and Henco, K. (1980) Int. J. Biol. Macromol. 2, 122-128.