Monoclonal antibodies to double-stranded RNA as probes of RNA structure in crude nucleic acid extracts

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ABSTRACT

We describe four monoclonal antibodies (MAB) which specifically recognize double-stranded RNA (dsRNA) together with their use in new methods for detecting and characterizing dsRNA in unfractionated nucleic acid extracts. The specificity of the antibodies was analyzed using a panel of 27 different synthetic and naturally occuring nucleic acids. All four antibodies reacted in a highly specific manner with long dsRNA helices, irrespective of their sequence; no binding to single-stranded RNA homopolymers or to DNA or RNA-DNA hybrids was observed. The apparent affinity of the antibodies to short (≤11bp) RNA helices was very low in all test systems used: only background levels of binding were obtained on single-stranded RNA species which contain double-helical secondary structures (e.g. rRNA, tRNA, viroid RNA). A sandwich ELISA and a dsRNA-immunoblotting procedure have been established which allow detection and characterization of dsRNA by MAB even in the presence of a large excess of other nucleic acids. In combination with temperature-gradient gelelectrophoresis (TGGE) not only the molecular weights but also the highly characteristic T_m-values of conformational transitions of individual dsRNA species could be determined by immunoblotting. An example of the general use of these methods for the detection of plant virus infections is demonstrated with groundnut rosette virus (GRV) dsRNAs. We were able to estimate the dsRNA content of infected leaves, identify the dsRNA species present in crude extracts and to determine the T_m-values of GRV dsRNA-3.

INTRODUCTION

The applicability of monoclonal antibodies as probes of nucleic acid structure *in vivo* and *in vitro* has already been shown in several systems (for review see 1–3). The production and characterization of structure-specific anti-nucleic acid antibodies is still a laborious and time-consuming procedure. However, in many cases, they offer the only possibility of investigating nucleic acid structure in complex systems and of proving the existence—and therefore relevance—of nucleic acid structures which have been predicted on the basis of sequencing data and thermodynamic calculations. The aim of our studies was to produce monoclonal antibodies against the A-helix structure of double-stranded RNA for this purpose. dsRNA was chosen as antigen because of (i) its immunogenicity (4–5) and (ii) frequent occurence in a range of different, biologically important, RNA species and (iii) because of the potential diagnostic value of high-molecular weight (HMW) dsRNA-species in plant virus infections (6–8).

The practical applications which we set out to achieve were the detection of infection of plants by RNA viruses and differentiation between infecting viral dsRNA species on the basis of their different MWs and thermal stabilities. If dsRNA—i.e. A-helix—specific monoclonal antibodies are to be used for identifying and characterizing HMW dsRNA, it is essential to be able to rule out cross-reactivity with single-stranded RNAs containing short base-paired secondary structures. In the present paper we describe the antigen specificity of 4 monoclonal antibodies which show very little, if any, cross reactivity with ssRNA in all of our test systems. Using these antibodies methods have been established to identify, quantify and characterize dsRNA species in crude nucleic acid and in aqueous extracts. One of the methods can also be applied to determine the individual T_m-values of conformational transitions of different viral dsRNAs present in the same extract.

To test whether our methods are indeed suitable for detecting viral dsRNA in natural virus-host systems we analysed crude extracts of groundnut rosette virus (GRV) infected plants. The genomic RNA of GRV is an ssRNA of MW 1.5×10^6 (9). In purified dsRNA preparations from GRV infected plants showing chlorotic or green types of rosette symptoms three prominent (dsRNA 1–3) and several minor dsRNA species were detected. Two of the major dsRNAs, dsRNA-1 (4.6 kbp) and dsRNA-2 (1.3 kbp) both hybridize to the genomic ssRNA and probably represent its replicative forms (10). The highly abundant dsRNA-3 (900 bp) seems to be a satellite RNA which is probably

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responsible for the symptoms of groundnut rosette (10,11). By using dsRNA specific MAB we detected the same dsRNA species in aqueous leaf extracts as those described in the literature (10) and determined the thermal denaturation profile of dsRNA-3.

MATERIALS AND METHODS

Nucleic acids

(i) *Synthetic homopolymers*. Poly(G), poly(I), poly(dC), poly(dT) and poly(A)·poly(dT) were purchased from Pharmacia-LKB (Uppsala, Sweden); the other synthetic single-stranded nucleic acid homopolymers and poly(I)·poly(C) were obtained from Boehringer Mannheim GmbH (Mannheim, FRG). The hybrids poly(I)·poly(dC), poly(A)·poly(U), poly(U)·poly(dA), poly(dA)·poly(dT) and poly(G)·poly(dC) were prepared by annealing equimolar amounts of the two strands in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

(ii) *DNAs*. Herring sperm DNA (Boehringer Mannheim, FRG) was denatured by heating at 90°C for 10 minutes in TE buffer and rapidly chilled on ice. Supercoiled plasmid DNA (pKK3535 (12)) was kindly provided by G.Theißen, crude nucleic acid extract containing DNA as well as RNA of pine by E.Beuther.

(iii) *Naturally occuring ssRNAs*. *E. coli* 16S + 23S rRNA and tRNA were obtained from Boehringer Mannheim GmbH. Potato spindle tuber viroid (PSTVd), 5S RNA and 7S RNA of tomato were purified from nucleic acid extracts by HPLC as described (13). Two PSTVd strains were investigated, PSTVd-DI (14) and its artificial mutant $PSTVd_{318}$ (15), both of which contain several helical regions with a maximal length of 9 and 11 bp, respectively. *In vitro* transcripts of a dimeric PSTVd sequence were synthesized with T7 RNA−polymerase using pRH 721/EcoRI as template (16). In order to obtain the trihelical structure containing a particularly stable helical region (17) transcripts were heated to 90°C for 5 minutes and allowed to cool slowly to room temperature.

(iv) *Viral dsRNAs*. The double-stranded viral satellite RNAs peanut stunt virus associated RNA 5 (PARNA 5, 393 bp (18)) and cucumber mosaic virus associated RNA 5 (nCARNA 5, 335 bp (19)) were kindly supplied by J.Kaper. Genomic dsRNAs of reovirus Serotype 3 were a gift of H.Becht and H.Müller; the 10 genomic segments of reovirus range from 1.2 to 3.8 kbp (20). L-dsRNA, the 4.3 kbp large genomic fragment of yeast killer factor (21) was prepared from the yeast strain YNN27. Yeast cells were grown in YEP medium containing 10 g/l of yeast extract, 20 g/l enzymic hydrolysate of casein and 30 g/l glucose (22) at 30°C for one day. The medium was then adjusted to 3% ethanol (23) and the culture incubated for further 24 hours. Cells were harvested by centrifugation, washed twice in 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and disrupted in a glass bead homogenizer (Braun AG, Melsungen, FRG), using glass beads with a diameter of 0.45−0.55 mm. After centrifugation at 4000 rpm for 2 min the supernatant was adjusted to 0.5% sodium dodecylsulfate, phenolized twice and extracted by chloroform. Nucleic acids were precipitated by incubating with 0.5 volume of 30% polyethylene glycol in 1.5 M NaCl at 0°C for 30 minutes. The pellet was dissolved in STE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.1 and 1mM EDTA) in the presence of 15% (v/v) ethanol. dsRNA was isolated by CF 11-cellulose chromatography at room temperature as described (24).

Purified dsRNA from groundnut rosette virus infected peanut leaves was isolated as described (25). Aqueous extracts of infected peanut leaves were made by homogenizing with 1−3 volumes of TE buffer in a mortar. After centrifugation at 10,000 g for 15 min the extracts were stored at −70°C until use.

Immunization and hybridoma production

Five female DBA/2 mice were injected intraperitoneally with a mixture of 50 μg L-dsRNA and 75 μg methylated bovine serum albumin, emulsified in complete Freund's adjuvant. The same amount of antigen was injected 3 weeks later in incomplete Freund's adjuvant and 7 weeks after the first immunization without adjuvant. Two mice with high anti-dsRNA serum titers were boosted again 3 days before fusion. Spleen cells were fused with Sp2/0-Ag14 myeloma cells in the presence of polyethylene glycol as described (26). The culture supernatants were screened in AN-ELISA (see below) using L-dsRNA as antigen, and selected cultures were cloned twice by limiting dilution.

Enzyme linked immunosorbent assay (ELISA)

Two ELISA procedures were used to characterize the antigen specificity of our antibodies. First, different nucleic acid antigens were immobilized through electrostatic interactions on microtiter plates coated with the positively charged protamine sulfate (anti-nucleic acid ELISA, AN-ELISA (27)). Second, nucleic acid species supposed to contain epitopes with A-helical structure were selectively bound via specific interactions with the dsRNA-specific IgG antibody J2 (Sandwich ELISA).

1. *AN-ELISA*. Polyvinyl chloride microtiter plates (Microtest III, Falcon) were precoated at 37°C with a 2% (w/v) solution of protamine sulfate (research grade, Serva, Heidelberg, FRG) in PBS (0.15 M NaCl and 10 mM K-phosphate buffer, pH 7.2) for 2 h. Precoated plates were incubated at 37°C with 0.5−300 ng of nucleic acids dissolved in TE buffer for 2 h and washed three times with PBS containing 0.5% (v/v) Tween 20. Hybridoma supernatants or appropriately diluted sera were incubated with bound nucleic acid (37°C, 2 h), and the plates were washed again with PBS + 0.5% (v/v) Tween 20. Alkaline phosphatase labeled $F(ab')_2$-fragment of goat anti-mouse IgG (H+L) (Dianova, Hamburg, FRG) was added to detect antibody bound to nucleic acids by using p-nitrophenyl phosphate (100 μg/well) as substrate. Absorption was measured with a MR 700 Microplate Reader (Dynatech) at 405 nm.

2. *Sandwich ELISA*. Microtiter plates were precoated with 0.4 μg/well protein A at 4°C overnight. Free binding sites were saturated with 2% BSA in PBS and the plates were then washed, filled with PBS and stored at 4°C. The dsRNA-specific J2 monoclonal antibody (IgG2a) was immobilized onto the protein A layer by incubation of 100 μl hybridoma supernatant per well at 4°C overnight. The plates were washed three times with PBS plus 0.5% (v/v) Tween 20 and nucleic acid antigens were added in TE buffer (37°C, 2 h). After washing as above bound antigens were identified by successive incubation (1) with the 1:2 diluted hybrodoma supernatant of the dsRNA-specific K2 IgM monoclonal antibody and (2) with an alkaline phosphatase conjugated, 1:5000 diluted goat anti-mouse IgM (μ) secondary antibody (Dianova, Hamburg, FRG). Both incubation steps were carried out at 37°C for 1−2 h. Washing, substrate incubation and reading of absorption were as in AN-ELISA.

Gel electrophoresis

Nucleic acid extracts or purified dsRNAs were electrophoresed in conventional polyacrylamide-TBE gels (5% (w/v) polyacrylamide at a 30:0.8 acrylamide:bisacrylamide ratio, 89

mM Tris, 89 mM boric acid, pH 8.3 and 2.5 mM EDTA) or in a temperature-gradient gel system (TGGE, 28). TGGE was carried out in 5% polyacrylamide gels containing 8 M urea in 0.1×TBE electrophoresis buffer. The nucleic acids entered the gel at 300 V (20°C, 1 h), then a temperature gradient of 20 to 70°C was generated vertically to the electric field. The actual temperatures reached in the gel were measured with a thermistor (PT 100 FKG 4304, Heraeus, Hanau, FRG) directly after electrophoresis. The temperature values indicated in the figures are the measured values between which the temperature-gradient was linear. The electrophoresis was carried out at 500V for 2.5 h, then the gels were blotted or silver stained (29).

Table I: The reaction of nucleic acids tested with the monoclonal antibodies J2, J5, K1 and K2 in AN-ELISA. The AN-ELISA test was carried out as described under Material and Methods. The nucleic acid antigens were applied at 300 ng/well except for L-dsRNA which was used as a positive control at 1, 3 and 10 ng/well. Synthetic dsRNA homopolymers and viral dsRNAs were positive with all 4 MAB soon after 15 min, however, all other nucleic acids were negative even after 16 h of substrate incubation. The results indicate that the antibodies specifically recognize dsRNA structure. +: positive reaction, −: negative reaction. The results apply for all 4 MABs except were indicated. For quantitative data see also Table II and Fig. 2.

A. Synthetic nucleic acids

Synthetic ssRNA homopolymers	Synthetic ssDNA homopolymers
poly(A) −	poly(dA) −
poly(U) −	poly(dC) −
poly(G) −	poly(dG) −
poly(C) −	poly(dT) −
poly(I) −	
Synthetic dsRNA homopolymers	Synthetic dsDNA homopolymer
poly(A)·poly(U) +	poly(dA)·poly(dT) −
poly(I)·poly(C) +	
Synthetic RNA-DNA hybrids	*in vitro* transcripts of PSTVd
poly(A)·poly(dT) −	dimeric recombinant clones
poly(U)·poly(dA) −	
poly(I)·poly(dC) −*	
poly(G)·poly(dC) −	

B. Naturally occuring nucleic acids

DNA:	Herring sperm DNA (native and denatured)	−
	Supercoiled plasmid DNA (pKK3535)	−
DNA+RNA:	Crude nucleic acid extract of pine	−
ssRNA:	*E.coli* tRNA	−
	Tomato 5S RNA	−
	Tomato 7S RNA	−
	Potato spindle tuber viroid (PSTVd-DI and $PSTVd_{318}$	−
dsRNA:	L-dsRNA from *Saccharomyces cervisiae* strain YNN27	+
	Reovirus dsRNAs	+
	Cucumber mosaic virus associated RNA 5 (CARNA 5)	+
	Peanut stunt virus associated RNA 5 (PARNA 5)	+

*The antigen showed a very weak cross-reactivity with K2 (A_{405} nm, 16h = 0.1).

dsRNA immunoblots

Electrophoretically separated nucleic acids were transfered to the positively charged Zeta-Probe (Bio-Rad, Munich, FRG) or Biodyne B (Pall Bio-Support membrane, Dreieich) membrane in an LKB, 2005 Transphor blotting chamber (Pharmacia/LKB, Freiburg) in 1×TBE buffer at 0.8−1A for 2 hours. After electrophoretic transfer free binding sites on the membrane were saturated with 0.5% (w/v) blocking reagent (Boehringer, Mannheim, FRG) plus 50 μg/ml sonicated herring sperm DNA in PBS at 4°C overnight. On the next day the membrane was incubated with hybridoma supernatant at room temperature for 2 h, washed twice with PBS+0.1% (v/v) Triton X-100 (30 minutes, room temperature) and further incubated with alkaline phosphatase conjugated $F(ab')_2$-fragment of goat-anti-mouse IgG (H+L). After washing bound antibodies were localized by staining with 0.2 mg/ml α-naphtol-AS MX-phosphate and 1 mg/ml Fast Red in TBS buffer (50 mM Tris-HCl, pH 8.2 and 150 mM NaCl, (30)).

RESULTS

Production of dsRNA specific monoclonal antibodies

After the third immunization with L-dsRNA sera of five DBA/2 mice were tested in AN-ELISA using L-dsRNA and poly(A)·poly(U) as antigen. All sera were positive with both antigens at 1:100 dilution. Two sera, which were positive up to a dilution of 1:1000, were analyzed for cross-reactivity with the single-stranded RNA-homopolymers poly(A) and poly(U) and with native and denatured herring sperm DNA. Since no cross-reaction was found, the corresponding animals were selected for hybridoma production.

Two fusions were carried out and 4 stable hybridoma lines (J2, J5, K1 and K2) were established which produce monoclonal antibodies highly specific for dsRNA antigens (see below). The J2 and K1 antibodies belong to the IgG2a, J5 to the IgG2b and the K2 antibody to the IgM class; the light chains of all four antibodies are of the kappa type.

Characterization of antigen specificity by AN-ELISA

In order to characterize the specificity of monoclonal antibodies their binding to a large panel of different synthetic and naturally occuring nucleic acids was investigated. 27 potential antigens were tested, the list of antigens and their reactions are given in Table I. As a positive control we applied L-dsRNA (1−10 ng/well), a genomic segment of the yeast killer virus (21) which had been used for screening the antibodies during the cloning procedures. The other potential nucleic acid antigens were used at saturating concentrations (300 ng/well). To detect even low levels of binding O.D. readings were made up to 16 h after substrate addition. With naturally occuring dsRNAs and synthetic

Table II. Binding of MAB J2, J5, K1 and K2 to the synthetic dsRNA homopolymers poly(A)·poly(U) and poly(I)·poly(C) and to L-dsRNA. Binding was measured under the same conditions as in Table I. The absorption values given in the Table were recorded at 405 nm after 1 h and 16 h of incubation with substrate.

Antigen	ng NA added/well	J2		J5		K1		K2	
		1 h	16 h	1 h	16 h	1 h	16 h	1 h	16 h
poly(A)·poly(U)	300	0.589	2	0.459	2	0.740	2	1.652	2
poly(I)·poly(C)	300	0.520	2	0.440	2	2	2	2	2
L-dsRNA	1	0.058	0.152	0.075	0.199	---	---	0.036	0.083
L-dsRNA	3	0.144	0.521	0.138	0.485	0.026	0.060	0.121	0.410
L-dsRNA	10	0.356	1.413	0.320	1.109	0.114	0.484	0.448	1.820

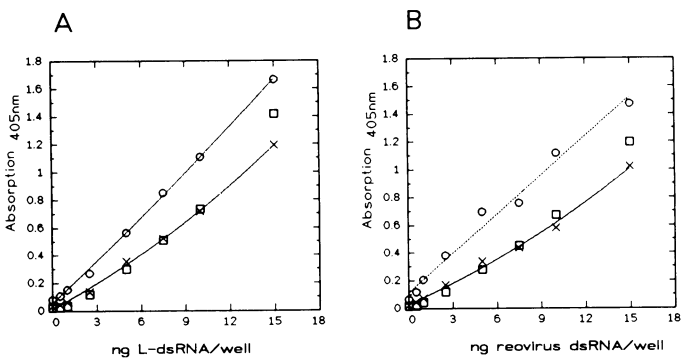

Fig. 1. Comparison of dsRNA binding to dsRNA specific MAB in the presence and in the absence of DNA, rRNA or tRNA by sandwich ELISA. L-dsRNA (1A) or reovirus dsRNA (1B) were used as antigens in the absence (-□-) or in the presence of 100 μg/well of herring sperm DNA (-×-) 1A and 1B), 16S + 23S rRNA from *E. coli* (-○-, 1A) or *E. coli* tRNA (-○-, 1B). The sandwich ELISA was carried out as described under Material and Methods and the absorption was measured 2 h after substrate addition. DNA, tRNA and rRNA showed no or only very low binding to the antibodies and they did not reduce the sensitivity of dsRNA detection. The results indicate that the apparent affinity of the anti-dsRNA antibodies to ssRNAs (and to DNA) may be several orders of magnitude lower than that to dsRNA.

dsRNA homopolymers a significant positive reaction was already obtained after 15 min incubation with substrate. The A_{405} values from these nucleic acids measured after 1 and 16 h are shown in Table II, where the viral dsRNAs are exemplified by L-dsRNA. At the same concentration as L-dsRNA the genomic segments of reovirus (1.2−3.8 kbp) gave signals nearly identical to those from L-dsRNA, the signals from the double-stranded satellite RNAs CARNA 5 (335 bp) and PARNA 5 (393 bp) were slightly lower (results not shown).

Even after an overnight incubation with substrate, no antibody binding could be detected with synthetic or naturally occuring ss- or dsDNA, with synthetic ssRNA homopolymers or synthetic RNA-DNA hybrids, except in the case of the K2 monoclonal antibody which showed a very weak ELISA signal (A_{405}=0.1) on poly(I)·poly(dC). Naturally occuring ssRNAs such as E.coli tRNA, tomato 5S and 7S RNA, potato spindle tuber viroid (PSTVd) and its *in vitro* transcripts—all of which are known to contain short intramolecular base-paired helices with lengths of up to 11 bp—also bound no detectable amount of any of the monoclonal antibodies.

Characterization of antigen specificity by sandwich ELISA

Because of the limited antigen binding capacity of the protamine sulfate coated plates used in AN-ELISA a sandwich ELISA procedure was established. In sandwich ELISA up to 100 μg nucleic acids per well were incubated on plates which were pre-coated with the dsRNA-specific IgG antibody J2. It was expected that nucleic acids containing dsRNA-epitopes would be selectively immobilized by J2 and positively identified by their binding to K2, which is also an dsRNA-specific MAB but belongs to the IgM class. Different naturally occuring dsRNA species were bound to J2 in the absence and in the presence of 100 μg/well DNA, tRNA or rRNA to determine the sensitivity of the method and to measure the cross-reactivity of the antibodies with the major nucleic acid components of the cell. From the results shown in Fig. 1 the following conclusions may be derived. (i) The sandwich ELISA method can detect dsRNA in the presence of a large excess (10^5 times by weight) of other nucleic acids and has the same sensitivity, ca. 1 ng dsRNA/well, as the AN-ELISA. (ii) DNA has no detectable affinity to our antibodies since practically the same absorption values were measured at all dsRNA concentrations with or without DNA added (Fig. 1A and 1 B). (iii) Both tRNA and 16S + 23S rRNA showed very low binding to J2 and K2 and were not able to saturate the binding sites on the J2-coated plates. The A_{405} value obtained with these antigens at 100 μg NA/well was 0.1 which is the same as that caused by 2 ng dsRNA (see intercepts on Fig. 1). As discussed later, this difference cannot solely be accounted for by the different number of epitopes per ng nucleic acid and may result from binding to tRNA or rRNA with an apparent affinity constant several orders of magnitude lower than that for dsRNA.

The ELISA data lead to the conclusion that the four monoclonal antibodies specifically recognize dsRNA structure and that the antigenic determinant is longer than 11 bp.

Differences in the fine specificity of the antibodies

The affinity of monoclonal antibodies to dsRNAs having different base compositions was investigated in AN-ELISA; the results are shown in Fig. 2. Poly(A)·poly(U), poly(I)·poly(C) or L-dsRNA were presented at concentrations varying between 1−300 ng/well and incubated with a constant amount of monoclonal antibodies. Under these conditions the K2 antibody bound equally well to all three antigens; however, the J2 and J5 antibodies showed a significantly higher affinity to L-dsRNA than to the synthetic dsRNA homopolymers. The largest difference in affinity was found with the K1 antibody which has a very high affinity to poly(I)·poly(C) but reacts weakly with poly(A)·poly(U) and L-dsRNA.

Monoclonal antibodies as probes for dsRNA on RNA-immunoblots: Sensitivity and specificity

The aim of the experiments described below was to identify and characterize dsRNA species in unfractionated nucleic acid extracts. Since dsRNA concentration in the extracts is expected to be low, the local concentration of dsRNAs was increased by electrophoresis under non-denaturing conditions in 5% polyacryamide gels. After electrophoresis the nucleic acids were electrophoretically transferred to a positively charged membrane and dsRNA was identified by using the monoclonal antibodies as specific probes (see Material and Methods). The sensitivity of the method was tested with different concentrations of purified L-dsRNA. As shown in Fig. 3A 60 pg dsRNA per band could still be detected using the J2 monoclonal antibody; the sensitivity is slightly higher than that of the silver staining. Detection of dsRNA by immunoblotting has already been reported (31), however, the sensitivity was 10^2-10^3 times lower than that of our procedure.

The binding of antibodies to short helical regions present in ssRNA molecules was also analysed by immunoblotting. Purified 5S RNA, 7S RNA or potato spindle tuber viroid (PSTVd) RNA from tomato plants was loaded in 5% polyacrylamide-TBE gels at concentrations of 10, 100 and 1000 ng/slot. After electrophoresis and blotting, filters were incubated with each of the 4 dsRNA-specific MAB or with a control antibody. The results obtained with PSTVd antigen are presented in Fig. 3B; the same reaction pattern was also observed with 5S and 7S RNA. No antibody binding could be detected using 10 or 100 ng PSTVd/slot. At an RNA concentration of 1 μg/slot, not only all four dsRNA specific MAB but also the control antibody 5/14,

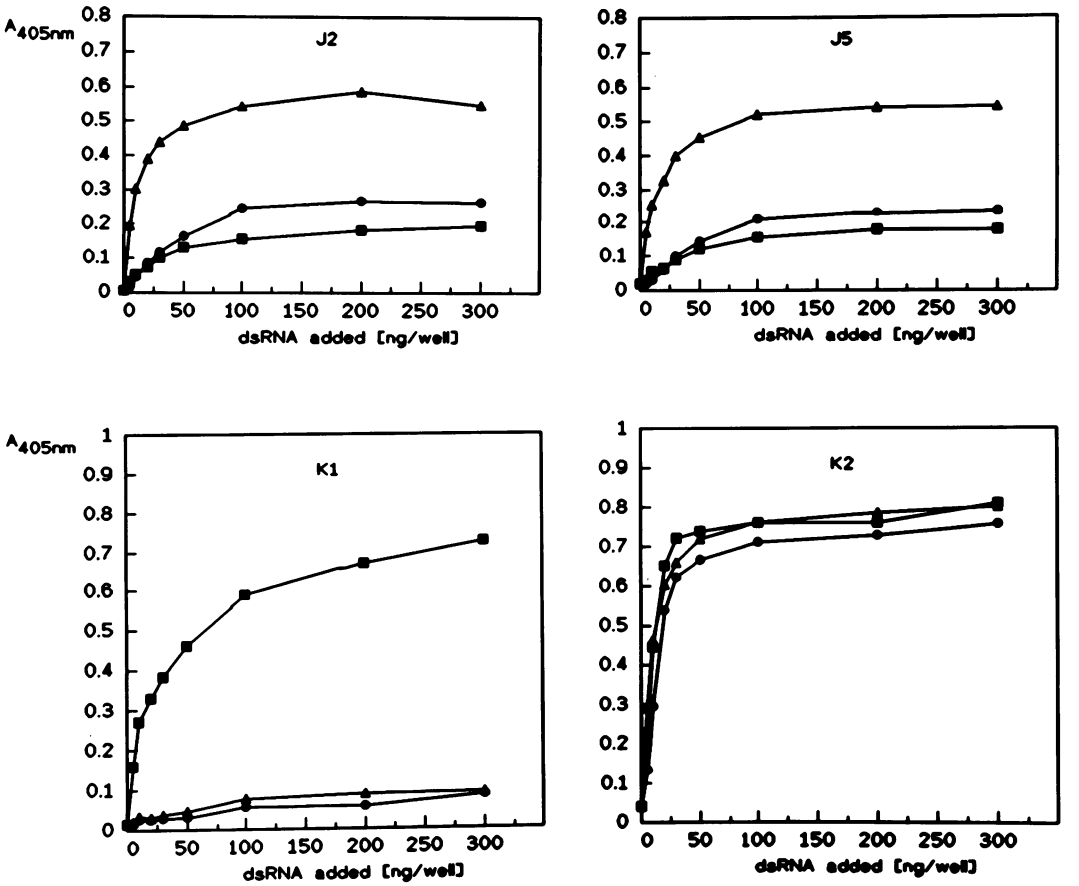

Fig. 2. Fine specificity of anti-dsRNA MAB. L-dsRNA (-▲-), poly(A)·poly(U) (-●-) or poly(I)·poly(C) (-■-) were used as antigens in AN-ELISA to detect quantitative differences in antibody binding to dsRNAs having different nucleotide compositions. The results show that although all antibodies mainly recognize dsRNA structure, the nucleotide composition and possibly the sequence of dsRNA may have an influence on the affinity of their binding.

which is specific for a coat protein of an animal herpes virus (32), showed a positive reaction. We interpret the latter finding as an unspecific binding caused by the high local antigen density. In agreement with the ELISA data the results suggest that our antibodies have only a very low affinity to the helices present in ssRNA antigens.

It follows from these results that using dsRNA-specific MAB in the sandwich ELISA and in immunoblotting procedures should allow the identification and characterization of dsRNA species in crude nucleic acid or aqueous extracts. This is demonstrated below on the example of groundnut rosette virus.

Detection and quantification of dsRNAs in groundnut rosette virus (GRV) infected plants

The dsRNA content of infected groundnut leaves was analyzed by sandwich ELISA. Leaves from 5 individual plants, each exhibiting a different time course of symptom development, were extracted in TE buffer 42 days post infection. After pelleting the cell debries appropriate dilutions of the supernatants were incubated with J2 antibody coated ELISA plates; purified L-dsRNA was used to obtain a calibration curve. As shown in Table III the estimates of the amount of dsRNA per g leaf tissue were very high (8–42 μg) in all plants tested, and no correlation between dsRNA content and symptom development could be detected 42 days after infection. The only possible exception was plant No. 5 which showed symptoms at the earliest time and harboured significantly more dsRNA than the others. According to our results the average concentration of dsRNA in GRV infected leaves is 19 μg/g tissue, i.e. 2–9 times higher than the dsRNA concentrations reported previously for plants containing double-stranded satellite RNAs (25,33). This discrepancy might be due to losses of dsRNA during purification in the latter cases. Since our sandwich ELISA allows detection of ng quantities of dsRNA, a few mg of plant tissue should be sufficient to detect and quantify GRV dsRNA in aqueous extracts of groundnut.

Table III. Estimated amount of dsRNA in GRV-infected leaves. Leaves were extracted 42 days post infection (p.i.) and the dsRNA-content of appropriately diluted aqueous extracts was determined by sandwich ELISA (see Materials and Methods). Purified L-dsRNA was used to obtain the calibration curve. n.d. = not detectable

		First symptoms (in days p.i.)	Estimated μg dsRNA/g leaf
GRV-infected Plant-No.	1	15	42
	2	24	8
	3	26	17
	4	29	16
	5	35	11
Non-infected Plant-No.	1	-----	n.d.
	2	----	n.d.

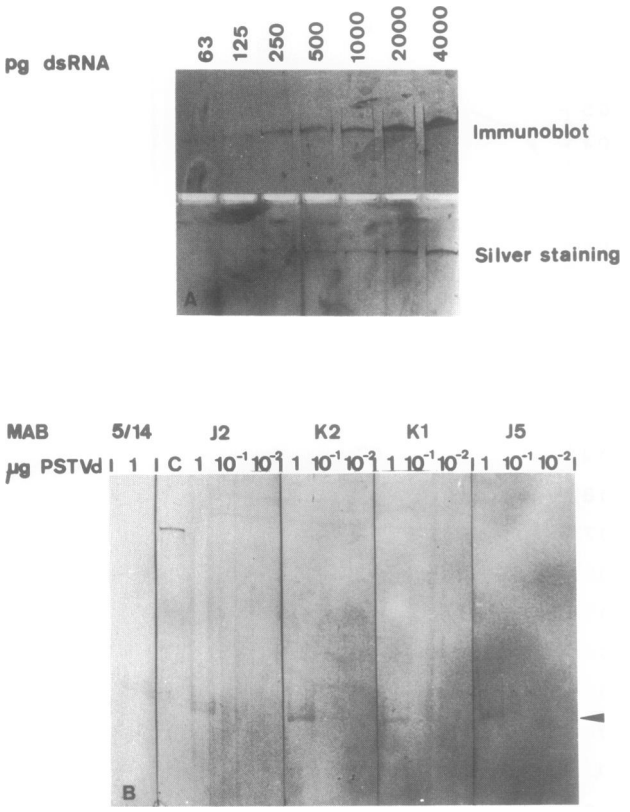

Fig. 3. Detection of dsRNA by immunoblotting: Sensitivity and occurrence of cross-reactivity at high ssRNA concentration. (A) The sensitivity of the method was tested by using L-dsRNA as antigen and J2 as primary antibody. We were able to detect as little as 63 pg dsRNA per band. Electrophoresis in non-denaturing 5% PAA gels, immunoblotting and silver staining were carried out as described under Material and Methods. (B) Binding of the J2, K2, K1 and J5 MAB to ssRNA was studied using viroid RNA (PSTVd-DI) at concentrations of 0.01 – 1 μg/lane. 1 ng L-dsRNA was used as positive control (lane C); the 5/14 MAB which specifically recognizes a pseudorabies virus glycoprotein (32) was used as negative control. Electrophoresis and immunoblotting conditions were the same as under (A). Cross-reactivity with all 5 MAB—independent of their specificity—occurs at 1 μg PSTVd/lane.

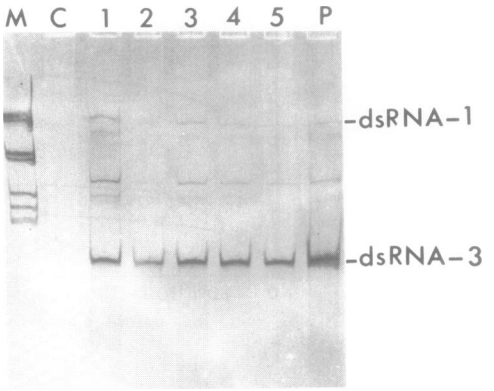

Fig. 4. Detection of dsRNA species present in GRV infected leaves. Purified dsRNA (P) and aqueous extracts from 5 different infected (1 – 5) and one control (C) plant were electrophoresed in 5% PAA-1×TBE gels under non-denaturing conditions. Immunoblotting was carried out as in Fig. 3 by using J2 as primary antibody. The same dsRNA species were detected in aqueous extracts as in the purified dsRNA preparation. All dsRNAs represent replicative forms and replication intermediates of GRV genomic RNA and of its satellite RNA. On the basis of their relative abundance and electrophoretic mobility two of the major dsRNAs were tentatively identified as dsRNA-1 (RF of genomic RNA) and dsRNA-3 (highly abundant ds-satellite RNA) according to the nomenclature given in (10). Reovirus dsRNAs were used as MW markers (M; MW range 3.8 kbp to 1.2 kbp).

The dsRNA species contributing to the ELISA signals were identified by immunoblotting. Three major and several minor dsRNA species were present in the five aqueous extracts as well as in a purified dsRNA preparation (Fig. 4, lanes 1 – 5 and lane P). No dsRNA was detected in the non-infected control (Fig. 4, lane C). The dsRNA pattern obtained by immunoblotting was identical to the pattern described in (10), and two of the major dsRNA species were tentatively identified as dsRNA-1 and dsRNA-3 (Fig. 4). The most abundant species was dsRNA-3, a satellite-like RNA present in at least 10-fold excess to the other dsRNAs.

Determination of T_m-values of conformational transitions and detection of sequence heterogeneity of GRV satellite RNA

Temperature-gradient gel electrophoresis (TGGE) allows determination of T_m-values of individual dsRNA species even in the presence of other dsRNAs (34). We identified the T_m-values of conformational transitions of dsRNA-3 in a purified GRV dsRNA preparation by TGGE and silver staining, and investigated whether it was possible to obtain the same thermal denaturation profiles in crude nucleic acid or aqueous extract by combining TGGE and dsRNA-immunoblotting.

As shown in Fig. 5 the electrophoretic mobility of dsRNA-3 changes dramatically between 41 °C and 46 °C (Fig. 5B). Three conformational transitions can be identified with T_{m1} = 42 °C ± 2 °C, T_{m2} = 43 °C ± 2 °C and T_{m3} = 46 °C ± 2 °C, respectively. Since the difference between T_{m1} and T_{m2} is only 1 °C these transitions are not always well resolved, probably because of slight variations in the slope of the temperature gradient between individual experiments. A thermal denaturation profile identical to that in Fig. 5B was obtained when the purified dsRNA sample was supplemented with 100 μg crude nucleic acid extract from healthy pine and dsRNA was identified by immunoblotting after TGGE (result not shown). The finding indicates that the combination of TGGE and immunoblotting allows determination of structural transitions of dsRNA in crude nucleic acid extracts.

The methods were also applicable to aqueous extracts of GRV infected leaves and the melting curve obtained for dsRNA-3 was identical to that of the purified dsRNA (compare Fig. 5A and 5B). Because of the higher sensitivity of the immunoblotting method not only dsRNA-3 but also the other dsRNA species (see Fig. 4) were detected. In addition, beside the major dsRNA-3 component, minor components with altered thermal stability appear, indicating sequence heterogeneity of dsRNA-3. When the dsRNA concentration is increased, sequence heterogeneity of dsRNA-3 can also be detected in the purified dsRNA preparation by silver staining.

DISCUSSION

Structural aspects of nucleic acid recognition by antibodies

We set out to produce monoclonal antibodies which specifically recognize the A-helix structure of dsRNA and which may therefore be used as structural probes to identify dsRNA *in vitro* and *in situ*. It is known from the literature that dsRNA is a reasonably good immunogen (4,5). However, polyclonal sera

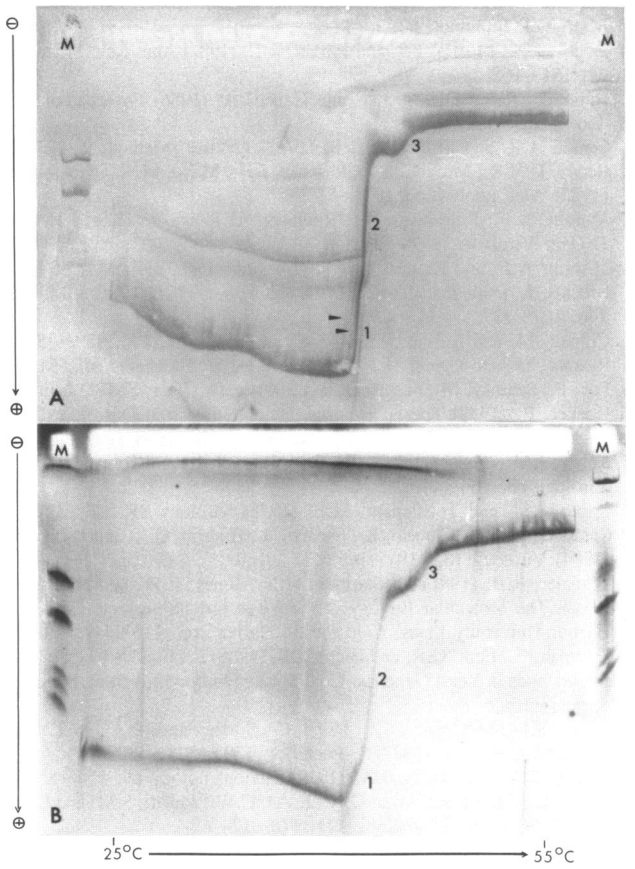

Fig. 5: Thermal stability of dsRNA-3 of GRV. Aqueous leaf extract containing 800 ng dsRNA (A) or the same amount of purified GRV dsRNA (B) was analyzed by TGGE in 5% PAA-1×TBE gels containing 8 M urea. dsRNA was identified by immunoblotting (Fig. 5A) or by silver staining (Fig. 5B). dsRNA-3 which is present in at least 10-fold excess to the other dsRNAs was detected by both methods and essentially identical melting curves of dsRNA-3 were obtained in the aqueous and the purified samples. Three conformational transitions (1–3) were resolved but no total strand separation of dsRNA-3 was achieved under our conditions. By immunoblotting which is more sensitive than silver staining sequence heterogeneity of dsRNA (indicated by arrows) was also detected and the less abundant other dsRNA species (compare Fig. 4) became also visible. M: reovirus dsRNAs.

elicited by dsRNA consist of different antibody populations: besides RNA A-helix-specific antibodies, antibodies which react with RNA-DNA hybrids (A′-helix), or which recognize only the specific immunogen sequence have been detected in these sera (1, 35). Since we intended to use monoclonal antibodies to identify any naturally occuring dsRNA, we immunized with L-dsRNA, a genomic RNA of the yeast killer virus, and selected for antibodies which recognized dsRNAs having different nucleotide compositions. As documented in the results section, the four MAB we obtained bind specifically to at least 16 different naturally occuring dsRNA-species (L-dsRNA, 10 genomic RNA segments of reovirus, CARNA 5, PARNA 5 and dsRNA 1–3 of GRV) as well as to poly(A)·poly(U) and poly(I)·poly(C) and show no cross-reactivity with ssRNA, DNA or RNA-DNA hybrids. Accordingly we conclude that the antibodies recognize the A-helix of double-stranded polyribonucleotide complexes.

Although the dsRNA structure is necessary and sufficient for antibody binding, quantitative differences in affinity to dsRNAs having different nucleotide composition may exist (Fig. 2). When tested in AN-ELISA the K2 antibody bound equally well to natural dsRNA, poly(A)·poly(U) or poly(I)·poly(C), whereas J2 and J5 showed preferential binding to natural dsRNA and K1 to poly(I)·poly(C). The observed differences may be caused by deviations in the stereochemical configuration of the individual dsRNA helices or by additional specific interactions of antibodies with individual nucleotides. To our knowledge, up to now, the isolation of two dsRNA-specific MAB has been reported. One of these antibodies recognizes an antigen determinant which is only present in the particular dsRNA (ds(n)CARNA 5) used for immunization and not even on closely related sequence variants thereof (36). The other MAB, elicited by immunization with poly(A) · poly(U), recognizes dsRNA structure like to our antibodies but showes higher affinity to synthetic dsRNAs than to reovirus dsRNA and has a higher tendency to cross-react with non-dsRNA antigens than our antibodies (37).

Since many ssRNA species contain short helical regions the cross-reactivity of anti-dsRNA antibodies was systematically investigated by AN-ELISA, immunoblotting and sandwich ELISA by using tRNA, rRNA, 5S RNA, 7S RNA and PSTVd as antigens. Surprisingly, very little or no cross-reactivity could be detected with these antigens. By dsRNA-immunoblotting, for example, which allows detection of 60 pg L-dsRNA, no antibody binding was seen at 100 ng viroid RNA (Fig. 3). In other words, a nearly 2×10^3-fold excess by weight corresponding to a $4-5\times10^4$-fold molar excess of viroid RNA was not sufficient to reach detectability by our antibodies. The trivial explanation that the epitope density on L-dsRNA might be 2×10^3 times higher than that on PSTVd can be excluded. It was shown experimentally and by thermodynamic calculation that under the conditions used for immunoblotting intramolecular base pairing of viroid RNA leads to a secondary structure in which short double helices and small internal loops form an unbranched, rod-like structure. In PSTVd-DI strain and its mutant $PSTVd_{318}$ used in our experiments the longest helix consists of 9 and 11 bp, respectively (14,15). Assuming a minimal epitope size of 9 bp, i.e. one epitope per PSTVd molecule (359 b), and assuming that epitopes of the same size are continuously arranged along the L-dsRNA molecule would allow us to account for at most a twentyfold difference by weight because of the different epitope density. Similar arguments apply to the results we obtained by sandwich ELISA where the same signals were obtained by addition of 100 μg 16S–23S rRNA or tRNA as by 2 ng of dsRNA. On the basis of these results we conclude that only RNA molecules possessing double helical regions larger than 11 bp can be detected with our anti-dsRNA antibodies. A variety of different mechanisms might cause this phenomenon. For example, if the intrinsic affinity of monovalent antibody binding is very low, it is conceivable that only bivalent antibody binding can be detected. If so, at least two adjacent epitopes would need to be present in the antigen—which is much less probable in ssRNA than in dsRNA species because of the low density and irregular distribution of double helical regions in ssRNA. It is also possible that RNA helices shorter than one helical turn are distorted and the deviations from an ideal A-helix are too large to be tolerated by the antibodies.

Identification of dsRNA in crude nucleic acid extracts by using monoclonal antibodies

Whichever interpretation of the binding mechanism turns out to be correct, because of the high specificity with which these antibodies recognize dsRNA they can be used to identify and characterize dsRNA species in crude nucleic acid extracts. By

combining TGGE and dsRNA-immunoblotting not only the MW but also the characteristic T_m-values of conformational transitions can be determined (Fig. 5). It should be emphasized that in contrast to optical melting curves several coexisting dsRNAs can be analyzed in the same experiment.

As demonstrated on the example of groundnut rosette virus (GRV) the antibodies were successfully utilized to detect and characterize viral dsRNA in crude nucleic acid preparations as well as in aqueous extracts. By comparing the dsRNA pattern of a purified dsRNA preparation with that of the aqueous extracts we found that according to their molecular weight and thermal stability the dsRNA species in both samples were identical, i.e. double-stranded replicative forms (RF) and replication intermediates (RI) were also present in aqueous extracts. The presence of RF and RI in nucleic acid extracts of virus infected plants has been reported by many authors (for review see (6)). However, it is still debated whether they are formed under physiological conditions *in vivo* or arise as *in vitro* artefacts under the specific conditions used for the extraction of nucleic acids (38). In contrast to nucleic acid extraction procedures no attempt is made to disrupt pre-existing protein-RNA complexes and no concentrating steps are carried out during the preparation of aqueous extracts. Thus, our finding either indicates the presence of RF and RI in infected cells or shows that if the corresponding (+)- and (−)-RNA strands are present, the formation of viral dsRNA is extremely favoured even under mild extraction conditions. Since other results obtained by immunofluorescence or electron microscope studies also point to the presence of viral RF and/or RI *in vivo* (39,40), we favorize the former interpretation.

By analyzing the thermal stability of dsRNA-3 we detected sequence heterogeneity of this satellite-like RNA. dsRNA-3 is thought to be largely responsible for induction of visually different disease symptoms and different isolates of dsRNA-3 have already been characterized although their sequences are still not known (11). Comparative thermodynamic analysis of dsRNA-3 isolates by TGGE might allow (i) discrimination between individual isolates and (ii) a decision whether the natural isolates consist of homogenous or heterogenous sequences of dsRNA-3. A similar analysis has successfully been applied to two other ds-satellite RNAs, CARNA 5 and PARNA 5 (41).

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