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Double-stranded cucumovirus associated RNA 5: experimental analysis of necrogenic and non-necrogenic variants by temperature-gradient gel electrophoresis

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ABSTRACT

Cucumber mosaic virus (CMV) and peanut stunt virus (PSV) each contain a fifth major RNA in the size range of 334 to 393 nucleotides. This fifth RNA is a satellite capable of modulating the expression of viral disease symptoms. It is present in infected tissue in single-stranded and double-stranded form. Nucleotide sequence variants of the double-stranded CMV-associated RNA 5 (dsCARNA 5) and PSV-associated RNA 5 (dsPARNA 5) were analysed by temperature-gradient gel electrophoresis. Gels were 5% polyacrylamide, containing 8 M urea in 8.9 mM Tris-borate buffer, with temperature differences of 25-40°C establishing gradients either perpendicular or parallel to the direction of the electric field. For dsCARNA 5 two characteristic transitions were detected with increasing temperature: at temperatures between 40°C and 46°C a drastic retardation in electrophoretic mobility induced by partial dissociation of the duplex structure from the ends and at temperatures above 52°C an abrupt increase in mobility due to complete strand dissociation. dsPARNA 5 exhibited both transitions at up to 10°C higher temperatures and an additional retardation between the transitions mentioned. Seven different variants of dsCARNA 5, 4 necrogenic and 3 non-necrogenic, were analysed. Some showed only one single band, others gave rise to up to six well separated bands corresponding to six molecular species. From all experimental results a correlation between the temperature of the retardation transition and the necrogenicity of CARNA 5 was derived. The diagnostic application of the temperature-gradient gel analysis in agriculture, particularly for the use of non-necrogenic variants as biological control agents to impede CMV-infections, is discussed.

INTRODUCTION

Viral satellites are small nucleic acids that can replicate only in conjunction with the nucleic acids of certain viruses in certain hosts. They do not share any significant sequence homology with their specific "helper" viral nucleic acids. All plant viral satellites described thus far contain or consist of single-stranded RNAs ranging in size from about 250 to 1700 nucleotide residues (for reviews see references 1-3). Cucumber mosaic viral (CMV) satellites of which the sequences have been reported to date range in size from 334 to 368 nucleotides (4-8). They have been discovered as a fifth major RNA component among the RNAs encapsidated within CMV, and to

distinguish them from the viral RNAs they are referred to as CARNA 5 (= CMV-Associated RNA 5). The recent increasing interest in CMV satellites can be attributed in part to the fact that they have been shown capable of significantly modulating the disease symptoms of the helper viruses (2). The satellite's remarkably rapid replication at the expense of the virus suggests this phenomenon to be a type of biological parasitism of the most primitive kind. This type of molecular level parasitism is presently leading to new approaches in the biological control of virus disease (2,3,9, 10,11,12,13).

The naturally occurring CARNA 5 variants have been found to exhibit strikingly different biological properties; on the one hand they may amplify disease symptoms leading for instance to lethal necrosis in tomato, on the other they are able to attenuate disease symptoms in other host plants (14,15). At the present time the possibility to utilize CARNA 5 as a control agent of diseases caused by CMV in the field has only been exploited in China (12,13). In this biological control procedure seedlings are preventively inoculated with CMV and a symptom-attenuating CARNA 5. This establishes a steady-state low-level infection with virus, CARNA 5 and its double-stranded (ds) form, dsCARNA 5, spreading throughout the plant (16). It is important, however, to verify that during this process no CARNA 5 variants capable of causing lethal tomato necrosis are emerging (17). While during infection small quantities of viral dsRNA are produced, as expected, dsCARNA 5 accumulates in proportions far greater than needed for a replicative function, and sometimes exceeds the amounts of ssCARNA 5 within virions. Late in the infection the synthesis of dsCARNA 5 apparently overtakes the synthesis of viral RNA and of ssCARNA 5, which are both suppressed to relatively low levels (18,19). This accumulation of dsCARNA 5 has been related to a possible mechanism that rationalizes the disease attenuation observed (20).

Due to their stability, their relative ease of detection and isolation, polyacrylamide gel electrophoresis of dsRNAs has recently been used as a diagnostic tool for (+) stranded RNA plant viruses (21). Several properties of dsCARNA 5 in particular make these molecules even more suitable for diagnostic purposes. Their presence in relatively high copy-numbers in CMV infected tissue would increase diagnostic sensitivity. Furthermore, the physico-chemical properties of dsCARNA 5, which are known in some detail (22), show that their thermal stability (and thus their optical melting behavior) may be altered by changing a single or a few base pairs in their

nucleotide sequence (22,23,24). This would mean that with appropriately simplified experimental technique it should be possible to differentiate dsCARNA 5 nucleotide sequence variants, including those derived from necrogenic or non-necrogenic CARNA 5 variants.

In this report it will be shown that dsCARNA 5 preparations isolated from CMV-infected plant tissues can be analyzed with respect to the presence of different nucleotide sequence variants by the method of temperature-gradient gel electrophoresis, which was developed recently in this laboratory as a routine procedure (25). In addition to the dsCARNA 5 results, temperature-gradient gel electrophoretic analysis was also applied to dsPARNA 5 isolated from plant tissue infected with peanut stunt virus (another member of the cucumovirus group) and its 393 nucleotide satellite PARNA 5 (26). In the accompanying paper (24) the experimental results obtained in the present work will be compared with the results from optical melting curves and calculations based on the known sequences of the different molecules.

MATERIALS AND METHODS

dsCARNA 5 was purified as described previously (27) from plant tissues infected with different isolates or strains of CMV. Following below is a listing of the different dsCARNA 5 variants used in this work, the virus isolate they relate to, and a description (or reference) to the source and passage history of the virus (if known) prior to the infection from which each specific dsCARNA 5 preparation was obtained.

WT-dsCARNA 5: From an infection of the WT (Wisconsin tobacco) isolate of CMV (28) in several plant batches taken from a continuing serial passage line in Nicotiana tabacum L. cv. Xanthi nc. It is necrogenic to tomato.

D-dsCARNA 5: From an infection of CMV strain D (29) in the last plant batch taken from a continuing serial passage line in Xanthi tobacco. It is necrogenic to tomato.

1-dsCARNA 5: From an infection of CMV strain 1 (30) in the last plant batch taken from a continuing serial passage line in tomato (Lycopersicon esculentum Mill, cv. Rutgers). It is non-necrogenic to tomato.

Val 24-dsCARNA 5: From an infection of the Val (Valencia) 24 isolate of CMV in "N tobacco" (17). CMV-Val 24 is a Spanish isolate from melon (Cucumis melo), and was brought into the N tobacco serial passage line in Beltsville via intermediate passages in cowpea (Vigna unguiculata L. Walp., Black

Eye), N tobacco, and Chenopodium quinoa (17). It is non-necrogenic to tomato.

Yn-dsCARNA 5: From an infection of CMV strain Y in the 6th of a serial passage line in Xanthi tobacco (31), when Yn-CARNA 5 was the only detectable satellite associated with the infection. It is necrogenic to tomato.

S-dsCARNA 5: From an infection of CMV strain S in Xanthi tobacco that had been inoculated with a mixture of the CMV-S genomic RNAs 1,2,3 and gel-purified S-CARNA 5 (8). It is non-necrogenic to tomato.

S52-dsCARNA 5: R-CARNA 5 was isolated from CMV strain R (32) obtained originally from H. Lot in 1974. It was transferred to Xanthi tobacco 1 time and passaged 15 times in squash (Cucurbita peop L., cv. Caserta Bush) after which the R-CARNA 5 was isolated. R-CARNA 5 was added to the genomic RNAs of a virulent CMV strain from China as described elsewhere (9). The satellite containing strain obtained was called S52. It is not necrogenic in pepper, squash, and tobacco, and is used as a biological control agent in China (9). However, after two passages in squash and about ten passages in tobacco, S52 induces necrosis in tomato. S52-dsCARNA 5 as used in this work was isolated from tobacco after ten passages.

Gel Electrophoresis

The technique of temperature-gradient gel electrophoresis will be described in detail elsewhere (25). A horizontal slab gel (200 mm x 210 mm x 1 mm) on a film support (FMC Corp. Rockland, Maine, USA) is in thermal contact with a copper plate, but electrically insulated from the copper. Using two independent thermostats, the copper plate was cooled on one side and heated on the other. This permits establishment of a linear temperature gradient between 10°C and 80°C in the gel. In reference runs the linearity of the temperature gradient inside the gel was tested by thermoresistors; the deviation from linearity was smaller than 0.3°C everywhere in the gel. The gel was covered by a plastic film and a glassplate, thus preventing evaporation from the gel and drying out. The device for the temperature-gradient was adapted to a LKB-Multiphor (LKB, Sweden). The direction of the electrophoresis could be arranged perpendicular or parallel to the direction of the gradient. For electrophoresis perpendicular to the temperature gradient the sample was pipetted into a single slot (170 mm x 4 mm), for electrophoresis parallel to the temperature gradient 16 different samples could be analysed, each applied into 8 mm x 3 mm slots. Gels contained 5% acrylamide, 0.12% bisacrylamide, 0.08% TEMED, 8.9 mM TRIS, 8.9 mM boric acid, 0.24 mM EDTA, 8 M urea and 0.06% ammonium peroxodisulfate

for starting the polymerization. The RNA samples were in 8.9 mM TRIS, 8.9 mM boric acid, 2.5 mM EDTA. Between 1 µg RNA (e.g. Fig. 1) if several heavy bands are present, and 200 ng (e.g. Fig. 2c) if only one component was contained, were used for an electrophoretic analysis.

In a first step, the samples were allowed to enter the gel at a constant temperature of 10°C and a voltage of 100 V for 1.5 hours. After switching of the voltage the temperature-gradient was established and allowed to equilibrate for 15 min. The third step is the actual electrophoretic analysis in the presence of the temperature-gradient at a voltage of 500 V for 1.5 hours. Further details of the procedure are described by Rosenbaum and Riesner (25).

The silver-staining of the gel originally described for proteins by Sammons et al. (33) was modified (34,35). The gel was slowly shaken twice for 5 min in 10% ethanol, 0.5% acetic acid, then for 15 min in 10 mM $AgNO_3$, followed by four times for 15 seconds in distilled H_2O. Subsequent treatment was for not more than 10 min in a fresh solution of 375 mM NaOH, 2.3 mM $NaBH_4$, 0.4% formaldehyde (37% W/V) and finally for 5 min in 70 mM Na_2CO_3.

RESULTS

Analysis of WT-dsCARNA 5

Fig. 1 represents the gel electrophoretic analysis of WT-dsCARNA 5 with a temperature-gradient from 35°C to 60°C perpendicular to the electric field. All analyses have been carried out in the presence of 8 M urea in order to shift the transition temperatures into an experimentally convenient range. At low and high temperatures the bands are moving much faster than at intermediate temperatures. Whereas the bands appear fairly homogeneous at low and high temperatures, they clearly split up in the region of the transition from the fast moving to the slow moving forms. This transition is called retardation transition. At least six different bands are separated. The T_m-value of band 'a' is 42°C, that of band 'f' 45.2°C. It is clearly demonstrated that WT-dsCARNA 5 contains at least six different RNA-species. Below the band of the dsRNAs a faster moving band is visible in all figures. This band is from an impurity in the gel matrix because it was also present if the sample slot was filled only with electrophoresis buffer without a nucleic acid sample.

The interpretation of the two transitions in Fig. 1 is straightforward. In the accompanying paper (24) a quantitative description of the helix-coil transitions of the dsCARNA 5 and its effect on the gel electrophoretic

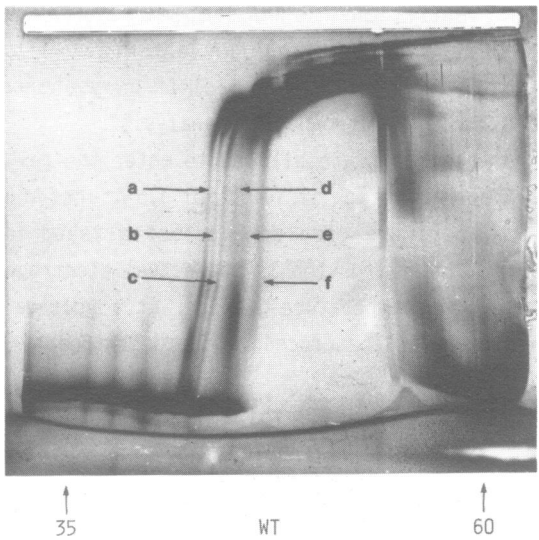

Fig. 1: Temperature-gradient gel electrophoresis of WT-dsCARNA5 with the gradient perpendicular to the electric field. At least six different RNA species, denoted a-f, are resolved at low temperature. The very broad and smeared bands in the region of low mobility are possibly an artifact from overloading. The arrows indicate the region of the linear temperature gradient.

mobility will be given. The drastic retardation in the first transition is due to the partial dissociation of the double strands from both ends. The subsequent abrupt increase of the mobility at high temperature is due to strand dissociation. Therefore, this second transition is called dissociation transition. Any single-stranded CARNA 5 contained in the sample besides the double-stranded CARNA 5 co-migrates at high temperature with the fast band seen in Fig. 1. The gel is heavily overloaded in the region of intermediate temperatures where the slow moving forms are present. The optimal concentration of the RNA is a compromise. It should not be too high because of possible overloading in the region of temperature-independent mobility and not too low because of the sensitivity of detection in the transition range.

From the gel analysis in Fig. 1 it cannot be deduced, which or how many of the six WT-dsCARNA 5 species are necrogenic. As a consequence of the multiplicity found it was tested whether the other CARNA 5 variants also consisted of different species and whether the different variants could be characterized by their electrophoretic behaviour in the temperature-gradient.

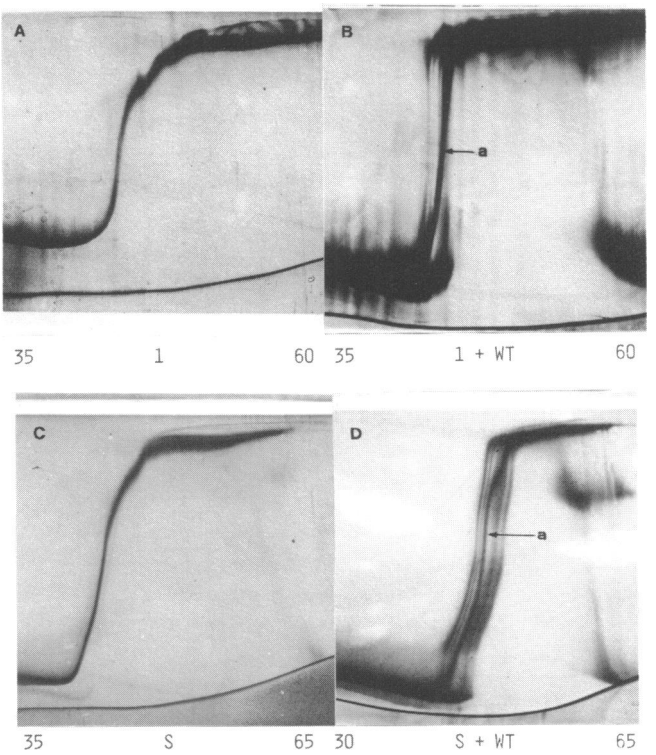

Fig. 2: Analysis as in Fig. 1 of non-necrogenic variants of dsCARNA 5. A: 1-dsCARNA 5, B: a mixture of 1-dsCARNA 5 (a) and WT-dsCARNA 5, C:S-dsCARNA 5, D: a mixture of S-dsCARNA 5 (a) and WT-dsCARNA 5.

Non-necrogenic variants 1-dsCARNA 5 and S-dsCARNA 5

The temperature-gradient gel analysis of 1-dsCARNA 5 (Fig. 2A) exhibits only one band although the gel had been overloaded on purpose. In clear contrast to WT-dsCARNA there is no indication for the presence of more than one molecular species. Strand dissociation is not detectable below 60°C. In order to compare the single component with the 6 components of WT-dsCARNA 5, a mixed sample of both was analysed (Fig. 2B). The heavy band of 1-dsCARNA does not coincide with any of the 6 of WT-dsCARNA 5. It is close to but different from band c of WT-dsCARNA 5. In the mixed sample of Fig. 2B, the bands of WT-dsCARNA 5 may serve as a temperature scale to determine with high relative accuracy ($\pm$ 0.1°C) the T_m-value of the transition of 1-dsCARNA 5.

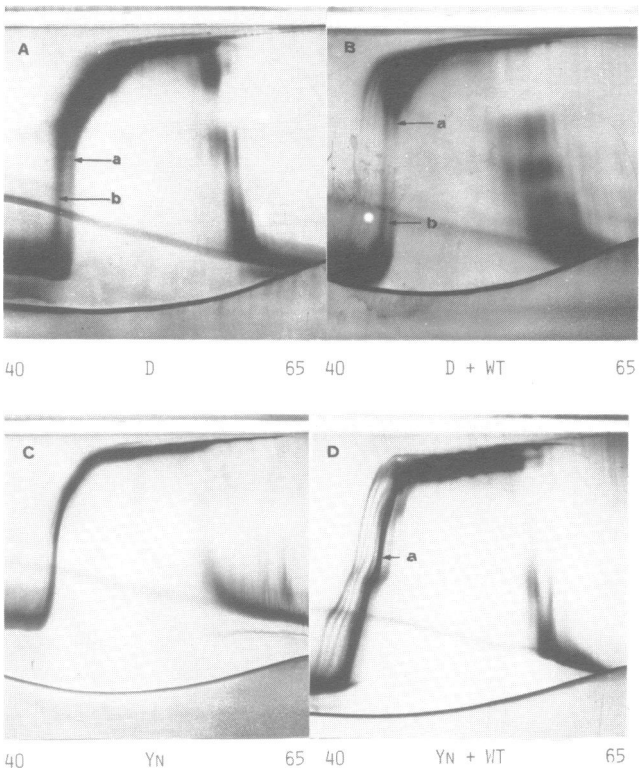

Fig. 3: Analysis as in Fig. 1 of necrogenic variants of dsCARNA 5. A: D-dsCARNA 5 with minor band (a) and major band (b), B: a mixture of D-dsCARNA 5 (a+b) and WT-dsCARNA 5, C: Yn-dsCARNA 5, D: a mixture of Yn-dsCARNA 5 (a) and WT-dsCARNA 5.

A similar analysis was also carried out for another non-necrogenic variant, S-dsCARNA 5. Similarly to 1-dsCARNA 5, only one band is detected with S-dsCARNA 5 (Fig. 2C); the T_m-value is also close to one of the less stable (lower T_m) RNA species of WT-dsCARNA 5, but it is different from that of 1-dsCARNA 5.

Necrogenic variants D-dsCARNA 5 and Yn-dsCARNA 5

From the variants which induce necrosis in tomato and of which the sequence is known, two were available for temperature-gradient gel analysis. In Fig. 3A and B the analysis of D-dsCARNA 5 alone and mixed with WT-dsCARNA 5 is depicted, Fig. 3C and D show the corresponding analysis of Yn-dsCARNA 5. From the preparation and sequence analysis D-dsCARNA 5 was

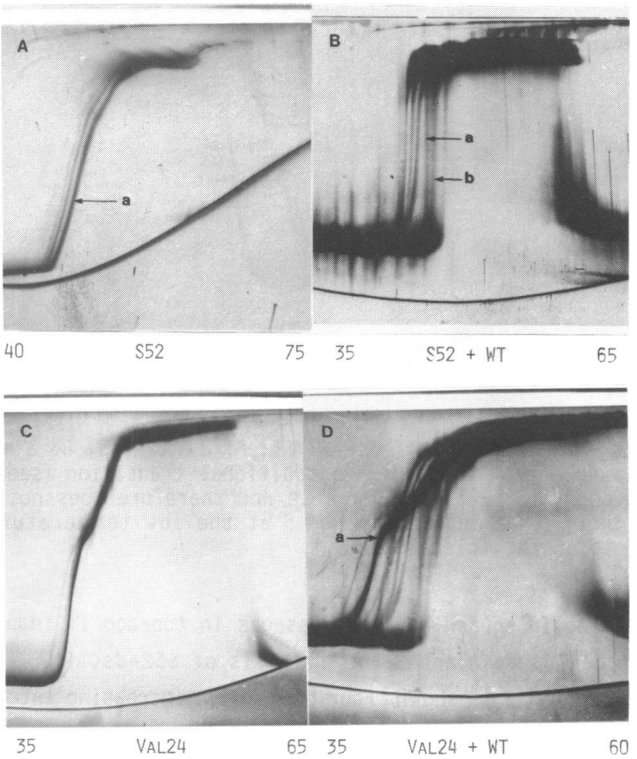

Fig. 4: Analysis as in Fig. 1 of S52-dsCARNA 5 and Val24-dsCARNA 5. A: S52-dsCARNA 5 with its dominant band (a), B: a mixture of S52-dsCARNA 5 and WT-dsCARNA 5. Comparison of Fig. 4B with Fig. 1 shows, that arrowed band (a), is band (a) in Fig. 4A, and arrowed band (b) is band e of WT-dsCARNA 5 in Fig. 1. C: Val24-dsCARNA 5, D: a mixture of Val24-dsCARNA 5 (a) and WT-dsCARNA 5.

expected to be homogeneous. In the gel analysis, however, a second band of minor intensity was detected. In the Yn-dsCARNA 5 a second band of much lower intensity (probably less than 1%) could also be detected. In comparing these necrogenic variants with the 6 components of WT-dsCARNA 5, their T_m-values of the retardation transition fall into the temperature range of the three more stable (higher T_m) components of WT-dsCARNA 5.

S52-dsCARNA 5 and Val24-dsCARNA 5

CMV S52 was constructed by combining the non-necrogenic R-CARNA 5 with a virulent CMV strain from China (see Materials and Methods). At first this new satellite containing strain was non-necrogenic in tomato; however,

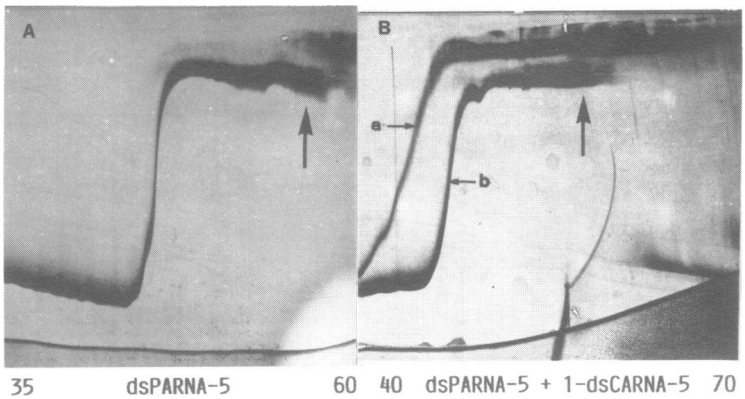

Fig. 5: Analysis as in Fig. 1 of dsPARNA 5. A: dsPARNA 5, B: a mixture of dsPARNA 5 (b) and 1-dsCARNA 5 (a). The additional transition (see arrow) in dsPARNA 5 (at about 55°C) is irreversible and therefore does not appear as a continuous band. The band of dsCARNA 5 at the low temperature edge is distorted due to an incomplete gradient.

after two passages in squash and ten passages in tobacco it induced necrosis in tomato. In Fig. 4A and B the analysis of S52-dsCARNA 5 alone and mixed with WT-dsCARNA 5 is shown. Four bands with increasing intensity were resolved going from lower to higher T_m-values. Characteristically, the band of dominant intensity is on the high temperature side of the 6 bands of WT-dsCARNA 5 in good accordance with the other necrogenic variants.

The non-necrogenic variant Val24-dsCARNA 5 was included in the studies, although its sequence is not known. It is an example of a qualitatively different band form in the temperature-gradient gel electrophoresis (Fig. 4C and D). The retardation transition is split into two steps the first of which is below the lowest transitions in WT-dsCARNA 5.

dsPARNA 5

The satellite PARNA 5 of peanut stunt virus contains about 60 nucleotides more than the satellite RNAs of CMV. In the temperature-gradient gel analysis (Fig. 5A and B) a second retardation transition around 55°C is obvious (see arrow). The first retardation transition and the dissociation transition are more stable than in 1-dsCARNA 5 (cf. Fig. 5B).

Multiple sample analysis in parallel temperature-gradient gel electrophoresis

If the temperature-gradient is applied to the gel in the same direction as the electric field, the RNA molecules start to migrate from the slot at

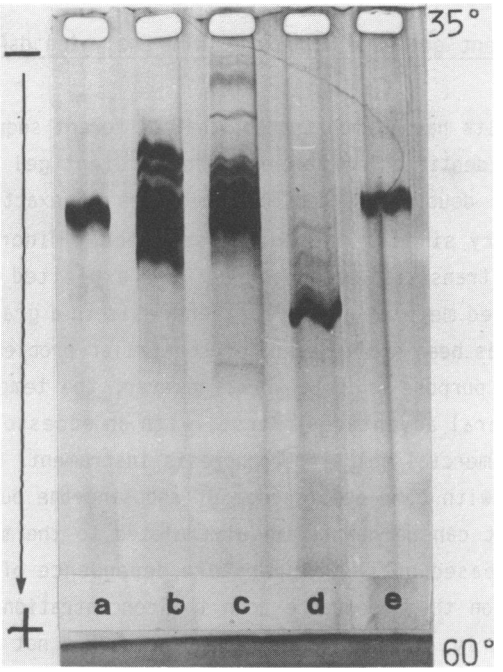

Fig. 6: Temperature-gradient gel electrophoresis with the gradient parallel to the electric field. The slots contain the following dsCARNA 5 variants: 1-dsCARNA 5 (a), WT-dsCARNA 5 (b), S52-dsCARNA 5 (c), dsPARNA 5 (d), Val24-dsCARNA 5 (e). The temperatures of the bands in this parallel gradient and the midpoint temperatures in the perpendicular gradient are only monotonically related but not linearly, because the exact shape of the transition has some influence. Deviating from the procedure given under Methods, the electrophoresis in presence of the temperature-gradient was carried out at 300 V for 1.5 hours.

low temperature, under native conditions. However, they are drastically retarded when they reach the temperature zone of their retardation transition. In separated slots, as in normal slab gel electrophoresis, several samples may thus be analysed simultaneously. The whole transition curve cannot be studied, but samples may be clearly characterized by their relative positions because they become retarded at specific temperatures. The gel electrophoresis has to be stopped before the samples reach the temperature range of strand dissociation and migrate faster again. A similar procedure can be carried out in an urea gradient (36). In Fig. 6 the analysis of several dsCARNA 5 species and of dsPARNA 5 is shown. The different positions and band multiplicities are in agreement with the analysis in the perpendicular temperature-gradient.

DISCUSSION

Temperature-gradient gel electrophoresis for analyzing dsRNA double helix-coil transitions

The above results have demonstrated, that different sequence variants of dsCARNA 5 may be identified by temperature-gradient gel electrophoresis although they are double-stranded RNA molecules of exactly or nearly the same size and very similar nucleotide sequence. Minor differences in double helix-coil transition temperatures are exploited in this type of analysis. A related method, e.g. electrophoresis in a gradient of a denaturing solvent, has been applied earlier to similar problems in DNA analysis (36). For the purpose of this work, however, the temperature-gradient analysis has several advantages: First, with an accessory it is easy to apply using a commercial gel electrophoresis instrument. Normal gel material may be used with complete freedom of choosing the buffer and solvent system. Second, it can be quantitatively related to thermodynamic theory, which is always based on the temperature dependence of the helix stabilities and not on the dependence upon the concentration of a denaturing solvent. Particularly, with RNA both dependencies are not linearly related (37). A temperature-gradient electrophoresis method of different design has previously been described in the literature (38). However, as far as we know it has not been applied to the type of problems described here.

The temperature-gradient electrophoretic analyses described show two types of transition: A retardation transition forming a continuous band at lower temperature and a dissociation transition displayed as a discontinuous increase in mobility at higher temperatures. As mentioned above and analysed quantitatively in the accompanying paper (24), the retardation transition represents an opening up of the double strand from the ends, whereas the transition at higher temperature is the strand dissociation. The first is a continuous transition, because melting without strand dissociation is fast compared to the time of the electrophoresis and the different RNA conformations are always in equilibrium. Strand dissociation at high temperature and particularly in low salt conditions, as in these experiments, is an irreversible or at least an extremely slow process. In addition, the gel matrix affects the process by inhibiting the dissociation as well as the re-association of the strands. Consequently, the gel analysis shows the equilibrium population of the non-dissociated state as a slowly migrating band and that of the dissociated state as a quickly migrating band. Thus, the very dispersed transition curve between the two states

is due to the irreversible dissociation of double helices during the electrophoresis.

Although the differences between different variants are bigger in the high temperature transition, this range is not appropriate for precise analysis because of the very dispersed transitions. The continuous transitions at lower temperatures are by far more favorable for precision. Particularly by using the 6 bands of WT-dsCARNA 5 as a background temperature scale, the midpoint-temperatures of the transitions may be determined with an accuracy of 0.1°C.

Correlation between stability and necrogenicity of dsCARNA5

Seven different preparations of dsCARNA 5 variants have been analysed. Whereas evidently WT-dsCARNA 5 was a natural mixture of six different species of comparable concentration, all other variants were either homogenous or nearly homogeneous (1, S, Yn, Val24) or yielded at least one fairly dominant band (S52 and D). The ssCARNA 5S of three of the latter six variants (1, S, Val24) are non-necrogenic and the other three (D, Yn, S52) are necrogenic in tomato. The different variants differ reproducibly in the stability of their retardation transition. The T_m-values listed in the Table show, that all non-necrogenic variants fall into the temperature range $T_m < 43.2$, the necrogenic variants in the temperature range $T_m > 43.8$. Although the apparent correlation between the necrogenicity property on the one hand, and the increased stability on the other, does not provide a molecular rationale for the necrogenicity of certain CARNA 5

Table: Denaturation temperatures (T_m) of the retardation-transitions of dsCARNA 5 variants determined by temperature-gradient gel electrophoresis.

variants	T_m (°C)
WT (a-f)	42, 42.35, 42.7, 43.7, 44.1, 45.2
1	42.9
S	43.2
Val24	40.5 (first step, 62% retardation) 44.2 (second step, 38% retardation)
D	44.1 (major band), 44.8
Yn	44.2
S52	43.9 (major band)

Buffer conditions are 8.9 mM TRIS, 8.9 mM boric acid, 0.24 mM EDTA and 8 M urea.

variants in tomato, the accompanying paper (24) suggests that the correlation also extends to certain nucleotide sequence differences found in necrogenic and non-necrogenic CARNA 5 species of which the nucleotide sequences have been determined.

The potential practical usefulness of the correlation between increased stability (higher T_m) and necrogenicity in tomato is that temperature gradient electrophoretic analysis will detect the appearance of such variants during serial propagation passages of CMV and CARNA 5. This is examplified with S52-dsCARNA 5, the only variant analysed in this work which has a history of having been applied as "vaccine" for the protection of tomato and pepper plants against CMV in the field in China (9). Although originally conceived as an attenuated CMV constructed from the non-necrogenic R-CARNA 5 and a local helper CMV strain (9), after serial passage in tobacco this vaccine tested necrogenic in tomato, This has been confirmed by temperature gradient gel electrophoresis in this work (Fig. 4), where a mixture of four bands with the major band in the higher T_m-range characteristic for necrogenic dsCARNA 5 was found.

The phenomenon of necrogenic CARNA 5 emergence upon serial passage in tobacco is now well-known and can probably be ascribed to the existence of variant populations of CARNA 5 where in certain host plants specific variants of greater "stability" are selected and preferentially replicated (17). Gel electrophoretic methods that can detect the emergence of such variants have been described (30,31), but these analyze the ssCARNA 5 form. Because ssCARNA 5 lacks the well-defined molecular shape of the dsCARNA 5 molecule this method does not have similar resolution power; moreover, unlike the dsCARNA 5 molecule, ssCARNA 5 does not yet allow the theoretical development of a rationale for its behavior in gel electrophoresis.

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