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Translation of turnip yellow mosaic virus RNA *in vitro*: A closed and an open coat protein cistron

(plant viruses/eukaryotic messenger RNA/protein synthesis/wheat germ extract)

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ABSTRACT **Sucrose gradient centrifugation of heat-denatured RNA of turnip yellow mosaic virus permitted the isolation of five RNA classes with molecular weights ranging from 2.0 to 0.25 × 10^6. The infectivity was shown to be confined to an RNA molecule of molecular weight 2.0 × 10^6. No significant increase in infectivity was obtained by combination of the latter RNA with the RNA classes of smaller size. Translation *in vitro* of the RNAs of different size classes in a wheat germ cell-free system revealed that the infectious RNA (molecular weight 2.0 × 10^6) does not promote the synthesis of the coat protein of turnip yellow mosaic virus. Efficient production of this coat protein was found exclusively when the smallest RNA class (molecular weight 250,000) was used as a messenger. It is concluded that RNA molecules of turnip yellow mosaic virus of molecular weight 2.0 × 10^6 contain a closed coat protein cistron and that RNA molecules of molecular weight about 2 to 3 × 10^5 with an open coat protein cistron can be isolated from the virions.**

It is generally believed that the infectious RNA of turnip yellow mosaic virus (TYMV) consists of a single uninterrupted chain with a molecular weight (M_r) of 1.9 × 10^6 (1–4). A mild heat treatment of freshly prepared TYMV RNA, however, gives rise to a considerable amount of smaller RNA pieces (4). It is assumed, therefore, that part of the virions contain RNA with one or more so-called "hidden breaks."

Various authors (1–4) observed occasionally discrete classes of smaller RNA molecules when denatured TYMV RNA was submitted to sedimentation in the ultracentrifuge. These molecules were ascribed to cleavage of the intact chain at specific sites, vulnerable to nucleases due to the secondary and/or tertiary structure of the RNA (3). The occurrence of RNA subunits has also been envisaged (1, 2).

We have been struck by the reproducible sedimentation pattern of heated TYMV RNA when centrifuged in sucrose gradients (4). The latter technique permitted the separation and isolation of five RNA classes with M_r estimated at about 2.0, 1.3, 1.0, 0.5, and 0.25 × 10^6.

In the present study we have asked ourselves the question: Does all infectivity reside exclusively in the 2.0 × 10^6 M_r fraction as was reported earlier (1), or is optimal infectivity obtained by combining fractions? To the best of our knowledge the latter possibility has not been entertained, but it is certainly not excluded since the earlier results were obtained with poorly resolved fractions. Moreover, a number of plant viruses contain a divided genome (5). The present data show, however, that infectivity is found in the highest size class of TYMV RNA and that no significant increase in infectivity is obtained by combining this RNA class with others. It may be concluded that all genetic information is present in TYMV RNA with a M_r 2.0 × 10^6.

When the latter RNA is used as a messenger for translation in a cell-free system of wheat germ, however, little or no coat protein was found among the biosynthetic products. In agreement with results of Benicourt and Haenni (6), unfractionated TYMV RNA yielded coat protein as the main biosynthetic protein. Screening of the various TYMV RNA classes mentioned above revealed that messenger activity for synthesis of coat protein was confined to the smallest RNA molecules only (about 0.25 × 10^6 M_r). It is concluded that full-size TYMV RNA (M_r 2.0 × 10^6) contains a closed coat protein cistron.

Abbreviations: TYMV, turnip yellow mosaic virus; M_r, molecular weight.

MATERIALS AND METHODS

Isolation of TYMV, TYMV RNA, and TYMV Coat Protein. TYMV was grown on Chinese cabbage (*Brassica pekinensis*, var. Witkrop). The virus was isolated by the method of Dunn and Hitchborn (7), and was finally suspended in 0.01 M Na acetate buffer, pH 6.0 and 10^{-3} M Na azide and stored at 4°. TYMV RNA was isolated by phenol extraction (8) in the presence of bentonite (1 mg/ml) and stored at −20°. TYMV coat protein was extracted according to Benicourt and Haenni (6).

Fractionation of TYMV RNA by Sucrose Gradient Centrifugation. RNA samples (1.0 ml) were layered on top of linear gradients of 15–30% sucrose in 0.1 M Na acetate buffer, pH 6.0 and centrifuged at 4° and 25,000 rpm in a Beckman SW-27 rotor. A capillary was placed on the bottom of the tube and the gradient was pumped through a flow cell of a Gilford 2000 spectrophotometer and monitored continuously for absorbance at 260 nm. Fractions of about 1 ml were collected and pooled as illustrated in the figures. The RNA was recovered by precipitation with ethanol and resuspended in Na acetate buffer, pH 6.0.

Infectivity Assay of TYMV RNA. TYMV RNA (0.020 ml of a solution containing 10–30 μg/ml) was assayed for infectivity on half leaves of Chinese cabbage. The plants were grown in composted soil in a greenhouse under artificial light conditions. To obtain local lesions after inoculation with TYMV RNA it is essential to omit fertilizer and keep the plants under high-pressure mercury lamps. Further optimalization occurs by keeping the plants in the dark 1 day before inoculation. After 5–6 days, local lesions were counted. A linear relationship between lesion number and amount of RNA tested was observed for the concentration range mentioned above. This infectivity assay will be described in more detail elsewhere.

Protein Synthesis in a Wheat Germ Cell-Free System. The preparation of the wheat germ cell-free extract was essentially the same as that described by Marcus *et al.* (10, 11) and Davies and Kaesberg (12). Wheat germ (General Mills Inc., Vallejo, Calif.) was purified by flotation on a mixture of cyclohexane and carbon tetrachloride (13). Dry embryos (1.0 g) were ground with sand (1.0 g) in a mortar in a total volume of 6.0 ml of 1 mM Mg acetate, 2 mM $CaCl_2$, 90 mM KCl, and 6 mM $KHCO_3$. The embryos were initially ground in 2.0 ml, with 2.0-ml increments

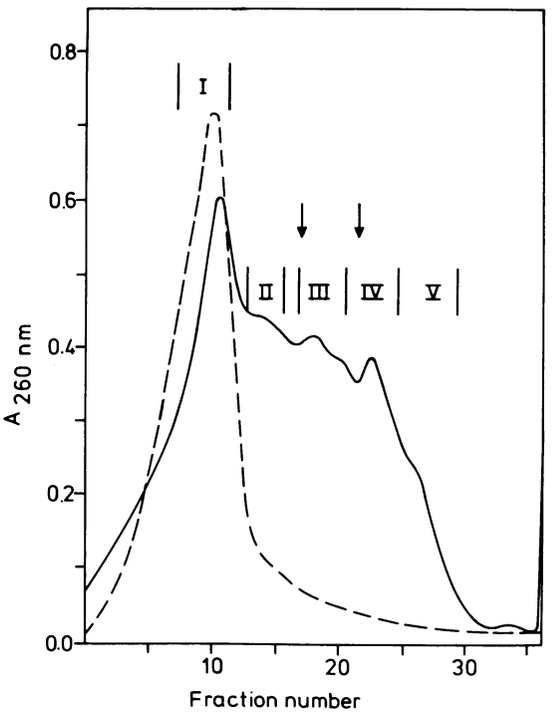

FIG. 1. Sedimentation of TYMV RNA in a sucrose gradient. For experimental details see *Materials and Methods.* (- - -) Unheated TYMV RNA (0.5 mg/ml); (—) TYMV RNA heated at 65° for 3 min before centrifugation. The arrows give the position, in a sister tube, of 23S and 16S rRNA of *Escherichia coli.* The gradient was fractionated as indicated (I–V). Sedimentation is from right to left.

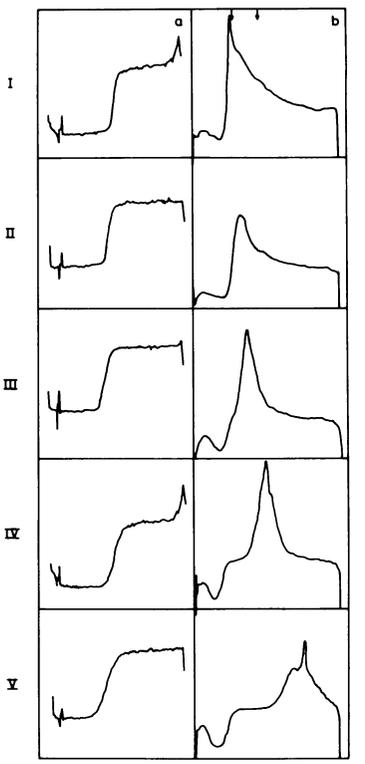

FIG. 2. Analytical ultracentrifugation and polyacrylamide gel electrophoresis of TYMV RNA classes I–V. Purification of the RNA classes was by two cycles of sucrose gradient centrifugation. (a) Analytical ultracentrifugation. RNA samples (70 μg/ml) in 0.01 M Na acetate, pH 6.0, were centrifuged at 48,000 rpm and 20° in a Spinco model E centrifuge. The UV absorbance scans were made at different times after maximal speed was reached. (b) Polyacrylamide gel electrophoresis. The procedure of Loening (15) was used with a modification. About 0.5 ml of formamide was layered on the gel. After electrophoresis for 1 hr the formamide was removed. Ten microliters of an RNA solution in 0.01 M Na acetate, pH 6.0, were mixed with 80 μl of formamide, heated at 65° for 5 min, and placed on the gel (2.5% polyacrylamide). Electrophoresis was for 4 hr at 5 mA per tube. Densitometric scans of the gels were made at 260 nm. The arrows represent the position of 28S and 18S rRNA of HeLa cells (a gift of E. A. C. van Oortmerssen).

added subsequently. DNase I was added to a final concentration of 5 μg/ml. After centrifugation for 10 min at 23,000 × *g*, 0.01 volume of 0.1 M Mg acetate and 0.02 volume of 1 M Tris-acetate, pH 7.6, were added to the supernatant and the suspension was recentrifuged for 10 min at 23,000 × *g*. Pellicle material, the cloudy portion of supernatant fluid above the pellet, and the upper lipid layer were discarded. Just before use 0.6 ml of the supernatant (S 23) was passed through a Sephadex G-25 column (18.0 × 0.8 cm) equilibrated with a buffer containing 1 mM Tris-acetate, pH 7.6, 50 mM Mg acetate, and 4 mM 2-mercaptoethanol. Material was eluted with the same buffer.

For protein synthesis *in vitro* the reaction mixture of 100 μl contained 30 μl of S23, 25 mM Tris-acetate (pH 8.2), 70 mM KCl, 1 mM ATP, 25 μM GTP, 2.5 mM phospho*enol*pyruvate, 1 μg of pyruvate kinase, 2.25 mM dithioerythritol, 50 μM of each of the unlabeled amino acids, 3.2 mM Mg acetate, [^{35}S] methionine (4 μCi, specific activity 150 Ci/mmol), and 2.5 μg of TYMV RNA. After incubation for 45 min at 30°, trichloroacetic acid (7%) was added and the mixture was heated at 90° for 15 min. After cooling the mixture was passed through a GF/C filter, the filter was washed with trichloroacetic acid, 7% and the radioactivity was assayed.

Polyacrylamide Gel Electrophoresis of Biosynthetic Proteins. When labeled biosynthetic polypeptides were to be analyzed by polyacrylamide gel electrophoresis, the reaction mixture was further incubated for 15 min at 30° with RNase A (10 μg/ml), T1 RNase (3 units/ml), and 60 mM EDTA, followed by precipitation with 7% trichloroacetic acid. The pellet was washed with 7% trichloroacetic acid, precipitated with acetone, and dried. The pellet was taken up in sample buffer, and 20 μl was applied to the gel. Analysis occurred on 12.5% sodium dodecyl sulfate slab gels essentially according to the procedure of Laemmli (14). The gels were stained with Coomassie brilliant blue, dried after destaining, and autoradiographed on Röntgenfilm for 5 days.

RESULTS

The sedimentation of TYMV RNA centrifuged in a 15–30% sucrose gradient containing 0.1 M Na acetate, pH 6.0, is illustrated in Fig. 1. Untreated RNA sediments for the greater part as one single peak with an estimated S value of 30. After a mild heat treatment (65° for 3 min), about 40% of the RNA sediments at the original rate (peak I), while the remainder sediments more slowly, giving rise to a number of peaks and shoulders which have been designated II–V. Occasionally, an extra peak may be observed between III and IV. Fractions of each peak were pooled and heated again at 65°. The material in the pools was purified separately by recentrifugation in sucrose gradients. The five RNA fractions thus obtained were analyzed in the analytical ultracentrifuge and by polyacrylamide gel electrophoresis (Fig. 2). Based on the moving boundaries of Fig. 2a, sedimentation coefficients were computed for RNA I–V. Although the electrophoretic analysis reveals considerable heterogeneity (Fig. 2b), an estimate of their molecular weights is given in Table 1.

Table 1. Molecular weight of TYMV RNA (classes)

Class	Sedimentation coefficient* (Svedberg units)	Molecular weight† ($\times 10^{-6}$)
I	21.1 ± 1.0	2.0
II	16.3 ± 0.6	1.3
III	14.5 ± 0.3	1.0
IV	11.6 ± 1.2	0.5
V	7.8	0.25

* Solvent is 0.01 M Na acetate at pH 6.0. The RNA (70 μg/ml) was centrifuged at 20°.
† Estimated from S-values and electrophoretic mobilities (see Fig. 2).

The infectivity of TYMV RNA was determined by counting local lesions on Chinese cabbage. Fig. 3 shows the infectivity distribution after one centrifugation, and Table 2 the infectivity of the purified fractions and combinations thereof. It follows from these data that RNA class I was more infectious than an equal amount of native, unfractionated RNA, heated or unheated. Occasionally, the number of lesions of class I was equal to that of the native RNA but never lower. The infectivity of RNA III–V was very small, but never zero. The number of lesions found in fraction II may be due to cross contamination with fraction I or to the presence of unfolded RNA chains with M_r 2.0×10^6. Combinations of fraction I with an equal amount of other fractions did not enhance the infectivity significantly. In some cases, however, a nearly 2-fold stimulation was observed (see Table 2). This was also observed when combinations of fractions from the sucrose gradient (Fig. 3) were tested.

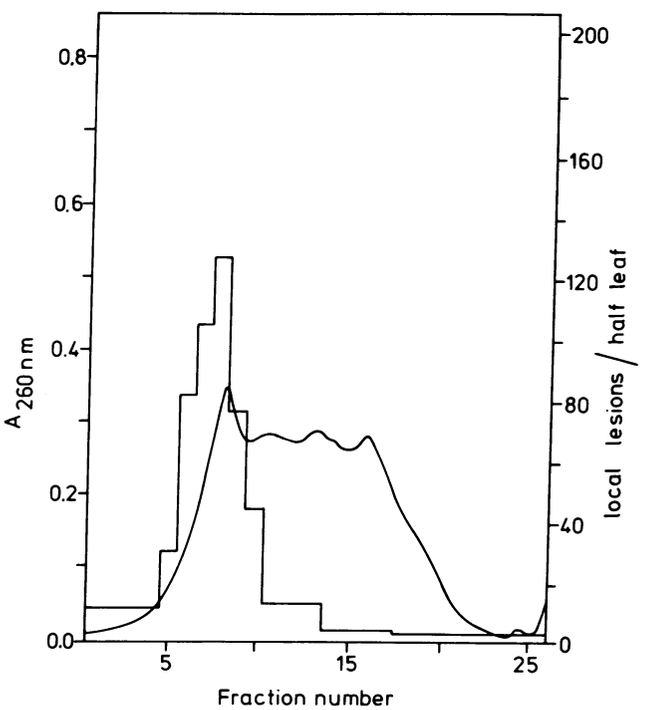

FIG. 3. Infectivity distribution of TYMV RNA in a sucrose gradient. A solution of TYMV RNA (1.0 mg/ml) was heated at 65° for 3 min and layered on a 15–30% sucrose gradient in 0.1 M Na acetate buffer, pH 6.0. Centrifugation was for 18 hr at 25,000 rpm and 4° in a Spinco SW-27 rotor. The gradient was fractionated as indicated, and 0.02 ml of each fraction was assayed for infectivity on 10 half leaves of Chinese cabbage (see *Materials and Methods*). Absorbance profile (—); lesion number (⌐¬⌐).

Table 2. Infectivity of TYMV RNA

RNA	No. of local lesions*
Unfractionated, unheated	36
Unfractionated, heated 3 min at 65°	35
Class I	48
Class II	28
Class III	3
Class IV	2
Class V	5
Combinations of classes†	
I + II	68
I + III	65
I + IV	81
I + V	44
II to V	20
I to V	53

For further details see *Materials and Methods*.
* Mean value of at least 10 inoculated half leaves of Chinese cabbage.
† The concentration of each class in a combination mixture was 16 μg/ml, which is the same when assayed separately.

These observations rule out the possibility that more than one RNA class is needed for a successful infection. The early claim (1) that all infectivity is confined to the fastest sedimenting RNA is confirmed. Apparently, this fraction, containing predominantly full length TYMV RNA, must be endowed with all the genetic information. Surprisingly, however, translation of this RNA in a cell-free system of wheat germ did not yield coat protein as one of the major biosynthetic products. This is illustrated in Fig. 4, in which the biosynthetic polypeptides, as obtained with the various RNA classes as messenger, were analyzed by polyacrylamide gel electrophoresis in slab gels containing sodium dodecyl sulfate.

Recently Benicourt and Haenni (6) demonstrated that native, unfractionated TYMV RNA can thus be translated into a number of polypeptides, including coat protein, which even appeared as the predominant product. The latter was identified by fingerprint analysis and immunoprecipitation. Our electropherogram, illustrated in Fig. 4, track 2, is essentially the same as that published by Benicourt and Haenni (6). We also found the same optimal conditions in terms of K^+ and Mg^{2+} concentrations for this *in vitro* system.

The autoradiographs of the gels illustrated in Fig. 4 further show that no RNA of the fractions I–IV (tracks 3–6) acted as an efficient messenger for coat protein synthesis. Class V, however, yielded a heavy band in the position of the coat protein (track 7) as almost the sole product. The products obtained with RNA I are too numerous so that each cannot result from a separate cistron. Except for the coat protein, about the same products were found when unfractionated TYMV RNA was used as a messenger [in full agreement with Benicourt and Haenni (6)]. It is possible that premature termination of large polypeptides occurs in the wheat germ system, but faulty recognition of initiation sites or aberrant proteolytic cleavage may also be envisaged.

DISCUSSION

The results reported in this paper show that the infectivity of TYMV RNA resides in RNA with an apparent M_r of 2.0×10^6. This confirms the earlier findings of Haselkorn (1). Smaller RNA molecules, which arise upon thermal deaggregation of TYMV RNA, are not only noninfectious by themselves, but do

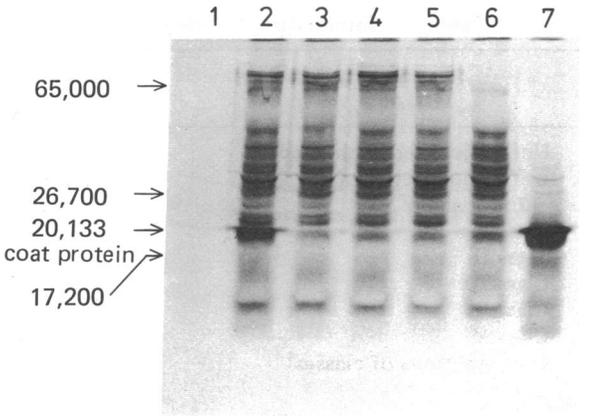

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S-labeled polypeptides synthesized under the direction of TYMV RNA. Each protein-synthesizing mixture (100 μl) contained 2.5 μg of RNA. It was found that RNA of size class I–IV yielded a similar incorporation of [^{35}S]methionine (350,000 cpm), whereas class V invariably showed an incorporation that was 1.5–2.0 times higher (600,000 cpm). The radioactive products were analyzed on 12.5% polyacrylamide gel by the procedure of Laemmli (14). Each sample (about 20 μl) layered on the gel contained the same amount of radioactivity (30,000 cpm). The position of some ribosomal proteins of 30S particles of *E. coli* and TYMV coat protein (M_r 20,133) used as markers is given. Track (*1*) no RNA added, (*2*) unfractionated TYMV RNA, (*3*) size class I, (*4*) size class II, (*5*) size class III, (*6*) size class IV, (*7*) size class V.

not enhance significantly the infectivity of the full-size molecules either. The nearly 2-fold stimulation that is found occasionally is considered to be nonspecific (16). It is still too early, however, to conclude from our experiments that the infectious units consist of uninterrupted RNA chains of M_r 2.0×10^6. It is possible that a heat treatment at 65° under conditions of moderate ionic strength is not sufficient for a complete deaggregation, while reaggregation on cooling must also be envisaged. In previous work (4, 8) we found that TYMV RNA shows a strong tendency to aggregate [possibly due to the high content of polyamines bound to the RNA or the high percentage of cytosine (38%)]. The use of a higher temperature during the heat denaturation (e.g., 80°) leads to a very rapid decrease of the infectivity of the RNA, probably caused by a thermal degradation of the RNA chain. No definitive conclusions can therefore be drawn concerning the integrity of infectious TYMV RNA molecules, although the findings of Bové and coworkers (17) concerning a TYMV-specific double-stranded RNA of 4×10^6 M_r strongly suggests an uninterrupted chain of 2.0×10^6 M_r.

From the present data it is clear, however, that the genetic information present in heat-resistant RNA molecules of 2.0×10^6 M_r is sufficient for successful infection. It was rather surprising, therefore, that the translation of this RNA in a cell-free extract from wheat germ does not result in the synthesis of coat protein. Apparently, the coat protein cistron on these molecules is not accessible for wheat germ ribosomes. A similar situation has recently been reported for the virion RNA of tobacco mosaic virus by Hunter *et al.* (18). During infection with this plant virus a small RNA is formed, the so-called LMC-RNA (for low-molecular-weight component; M_r about 250,000) (19, 20), which is readily translated into coat protein of tobacco mosaic virus by wheat germ ribosomes (18). It is tempting to suggest that a similar small RNA occurs in the case of TYMV, which can be isolated, as shown in the present paper, by fractionating heated TYMV RNA on sucrose gradients.

One can only speculate about the origin and the function of this coat protein messenger, which, according to its size (2 to 3×10^5 M_r) may be monocistronic [M_r of coat protein: 20,133 (21)]. Two possibilities may be envisaged with regard to its origin. (*i*) Specific cleavage of newly synthesized infectious RNA molecules yields a monocistronic messenger with an open coat protein cistron. Such a cleavage may occur either in the plant or the virion. The specificity of cleavage by a nuclease may be governed by the ordered secondary and/or tertiary structure of the RNA or by the specificity of the nuclease itself. (*ii*) Partial transcription of the negative strand of TYMV RNA may give rise to the coat protein messenger. When the messenger is not generated inside of the virion, encapsidation has to occur after its formation since active messenger has been isolated from a virion.

Two types of virions may be considered as possible sources of the coat protein messenger: (*a*) virions containing a full complement of RNA (about 35%). These virions, designated component B1 (22), should then contain RNA that partly consists of aggregates. This situation is feasible since a substantial proportion of native TYMV RNA usually contains so-called hidden breaks and thus behaves as aggregates with the same molecular weight as heat-resistant molecules of M_r 2.0×10^6. (*b*) Virions with lower RNA contents. The presence of such virions in preparations of TYMV has been demonstrated by Matthews (22, 23) and were designated B_0, B_{00}, and B_{000}. These minor components, at least B_0 and B_{00} which were also present in our virus preparations, do not stimulate the infectivity of B_1 (16). The origin and function of these particles are unknown, but the possibility exists that one of them contains the coat protein messenger.

The 3′ end of TYMV RNA with M_r 2.0×10^6 can be aminoacylated with valine (24). Recent studies (25) concerning the 3′-terminal nucleotide sequence of TYMV RNA revealed that the tRNA-like acceptor of valine is adjacent to the coat protein cistron.

In preliminary investigations (unpublished observations) we found a rather uniform distribution of valine acceptor activity per mg of RNA over the various TYMV RNA fractions (classes I–V). Apparently, class V RNA, which contains the active coat protein messenger, is not enriched in sequences containing the tRNA-like structure at its 3′ end. Polyacrylamide gel electrophoresis shows that this class of RNA is heterogeneous. It may be assumed, therefore, that further purification of class V RNA will yield an even more active coat protein messenger. This would also enable the determination of the structure of the 5′ end to see whether it is blocked by ^{7}mG as was shown to be the case for full-size TYMV RNA (C. W. A. Pleij, unpublished observations).

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