Volume 13 Number 19 1985 Nucleic Acids Research

Affinity electrophoresis for monitoring terminal phosphorylation and the presence of queuosine in RNA. Application of polyacrylamide containing a covalently bound boronic acid

Gabor L.Igloi and Hans Kössel

Institut für Biologie III der Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, FRG

Received 23 July 1985; Revised and Accepted 9 September 1985

ABSTRACT

An affinity electrophoretic method has been developed to study the state of terminal phosphorylation of RNAs and the presence of the hypermodified base Q in tRNA. It is based on the copolymerization of acryloylaminophenylboronic acid into standard polyacrylamide gels and the interaction of this derivative with free cis-diol groups present in the RNA. In the case of terminal phosphorylation, free ribose groups are present either as such, or may be introduced by enzymatic reactions specific for a particular phosphorylation pattern (e.g. using T4 RNA ligase or guanylyltransferase). Additionally, tRNA species containing the Q base may be resolved from Q-lacking tRNAs by boronate affinity electrophoresis. The introduction of a non-destructive, one-step electrophoretic procedure not only offers an alternative to classical analytical methods, but also provides a means of isolating such populations of RNAs for which other methods are unavailable or are less convenient.

INTRODUCTION

The state of nucleoside modification of RNAs is dependent on the extent of maturation which they have undergone. In this context, an examination of the structure of RNAs at the 3' and the 5' ends as well as within the nucleotide chain provides information concerning the pathways leading to their formation. For example, the 3' end of mature RNA carries a free ribose group but processing intermediates (1,2) or nucleolytic degradation products (e.g. 3) may be phosphorylated. The 5' end may be characterized by 5' hydroxyl, 5' monophosphate or, in the case of primary transcripts, di- or triphosphate groups. Translationally active mRNA may bear a 5' cap structure (4), while stable RNAs, especially tRNAs, are subject to internal post-transcriptional modification (5). In order to distinguish between these possibilities, it is standard practice to carry out a nucleolytic cleavage followed by an ana-

lysis of the digest resulting in the loss of the starting material. A non-destructive analytical method, or the possibility of fractionating all the various species would, therefore, seem to be desirable, but is not presently available.
We describe here a gel electrophoretic procedure with which a distinction between all possible terminally phosphorylated or RNA species containing the hypermodified base Q (6) may be achieved. It relies on the interaction during electrophoresis between a boronic acid derivative, covalently incorporated into the polyacrylamide gel, with free ribose groups in the RNA, either present as such in the material subjected to analysis or added to the RNA subsequently by specific enzymatic reactions (using T4 RNA ligase, guanylyltransferase or alkaline phosphatase). By making use of these reactions and the novel electrophoretic system, we have extended the concept of affinity electrophoresis (7,8) to the field of nucleic acids.

EXPERIMENTAL PROCEDURES

Synthesis of *N*-acryloyl-3-aminophenylboronic acid

1.86 g aminophenylboronic acid hemisulphate (EGA) was added in small portions to a rapidly stirring solution at $0^{o}C$ of 1.68 g $NaHCO_3$ in 40 ml deairated H_2O (boiling and cooling under vacuum). After the evolution of CO_2 had ceased 900 µl acryloylchloride (Fluka) in approximately 50 µl aliquots was dropped into the pale yellow solution over a 20-min period with the reaction maintained at $0^{o}C$. The product began to precipitate after about 2/3 of the acryloylchloride had been added. The resulting suspension was cooled to $-20^{o}C$ for 5 min, filtered under suction and the fawn solid was washed with small amounts of ice cold H_2O. The solid was dissolved in EtOAc and dried overnight with Na_2SO_4. The brown liquid was filtered off, the Na_2SO_4 washed with a few ml EtOAc and the washings combined with the main filtrate. Hexane was now added until no further cloudiness occurred and the resulting brown oil crystallized on cooling to $-20^{o}C$ overnight. The supernatant could be removed and the solid was recrystallized from H_2O to give 1.1 g (approx. yield: 70%) pale brown needlelike crystals (R_f = 0.17 on polyamide TLC, using H_2O:CH_3CN (9:1 v/v)). Extensive recrystallization from H_2O gave white needles,

while chromatography over a polyamide column (Polyamide CC6, Machery & Nagel) using a solvent of H_2O:CH_3CN (9:1 v/v) followed by lyophilyzation, gave a white powder. Elemental analyses reproducibly gave C, 53.49; H, 5.29; N, 6.92, which is consistent with the isolation of the product as a hemihydrate (Calc. C, 54.23; H, 5.02; N, 7.03). Incombustible material (up to 17%) hampered the analysis.

RNA Modifications

Periodate oxidation. Cleavage of cis-diol groups with periodate was carried out in a total volume of 50 µl containing the RNA in 10 mM NaOAc, pH 4.6, and 25 mM $NaIO_4$ for 60 min at 0°C in the dark. Separate tests on the amino acid acceptance of oxidized compared to non-oxidized $tRNA^{Phe}$ (yeast) (Boehringer) using an *E.coli* S100 extract under standard aminoacylation conditions (9), showed that all the accepting capacity of tRNA was destroyed by this treatment. The oxidation was stopped by the addition of 50 µl ethyleneglycol and a further incubation period of 30 min at 0°C in the dark. Before ethanol precipitation of the RNA, the excess $NaIO_3$, which is insoluble in ethanol, was removed by micro-dialysis (10) for 4 h at 4°C against 1 mM EDTA. Ethanol precipitation from 0.3 M NaOAc, pH 4.6, with 3 vol. ethanol yielded the RNA_{ox}. For the control, RNA was incubated with 10 mM NaOAc, pH 4.6, for 60 min at 0°C and then treated as above.

β-Elimination of the terminal base. tRNA-CCA_{ox} was incubated with a final concentration of 0.25 M lysine, pH 9.3, in a total volume of 100 µl at room temperature for 4 h in the dark. As control, tRNA-CCA_{ox} was treated with 0.25 M Tricine, pH 9.3, as above. To terminate the reaction, the pH was lowered to near 5 by the addition of one drop of glacial acetic acid and the tRNA was ethanol precipitated to give tRNA-CC_p.

Phosphatase treatment of tRNA-CC_p. The 3'-terminal phosphate remaining on tRNA-CC_p was removed by incubation with 0.2 M Tris-HCl, pH 8.0, 0.2 M $MgCl_2$ and 1 µg bacterial alkaline phosphatase (Boehringer) in a total volume of 50 µl at 37°C for 60 min. The 5'-terminal phosphate is, under these conditions, not digested (11). The tRNA-CC_{OH} was recovered by phenolization and ethanol precipitation.

5'-Adenylation using T4 RNA ligase

A DNA 30-mer prepared using an automated DNA synthesizer (Applied Biosystems) was labelled 5'-terminally with ^{32}P, by standard procedures. The oligomer, which had been ethanol precipitated in the presence of oxidized tRNA as carrier, was incubated with 50 mM Hepes-KOH, pH 7.8, 20 mM $MgCl_2$, 3.5 mM DTT, 10 µg/ml BSA, 10% Me_2SO, 1.5 mM ATP in a total volume of 50 µl. The reaction was initiated by the addition of 2 µg T4 RNA ligase (BRL) and continued at 37°C for 90 min. After phenol extraction, the DNA was ethanol precipitated.

Transcription *in vitro*

The 340 bp BglII-PvuI DNA fragment containing the RNA^{Val} gene from maize chloroplasts (12) was isolated and transcribed. Transcription was carried out at 37°C for 30 min in a medium (100 µl) containing 40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 0.8 mM KH_2PO_4 and 80 mM KCl. ATP, CTP and UTP were present at 0.2 mM and 50 µCi α^{32}-P GTP (> 400 Ci/mmol; Amersham) was added. The reaction was initiated with 1 µl (3 µg) RNA polymerase from *E.coli* (Miles). After the given time, 10 µl 10% SDS and 11 µl 3 M NaOAc, pH 4.6, was added, the mixture phenol extracted once and ethanol precipitated in the presence of 10 µg carrier tRNA. The main transcript was isolated by electrophoresis on a 10% acrylamide, 7 M urea gel followed by elution of the band with 0.3 M NH_4OAc - 20 mM Tris-HCl, pH 7.5, at room temperature overnight. The transcript was recovered by ethanol precipitation in the presence of 0.3 M NaOAc and 10 µg carrier tRNA.

Capping *in vitro*

The incubation mixture contained 50 mM Tris-HCl, pH 7.9, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 0.2 mM GTP together with ^{32}P-labelled RNA and 4 U guanylyltransferase (BRL) in a total volume of 50 µl. After 30 min at 37°C, the RNA was recovered by phenolization and ethanol precipitation in the presence of 10 µg carrier tRNA.

Affinity electrophoresis

An affinity electrophoresis gel was prepared by addition of the required amount of *N*-acryloyl-3-aminophenylboronic acid (APB) to the standard gel components. Thus, for a 10% acrylamide denaturing gel containing 2% APB (with respect to the total acrylamide

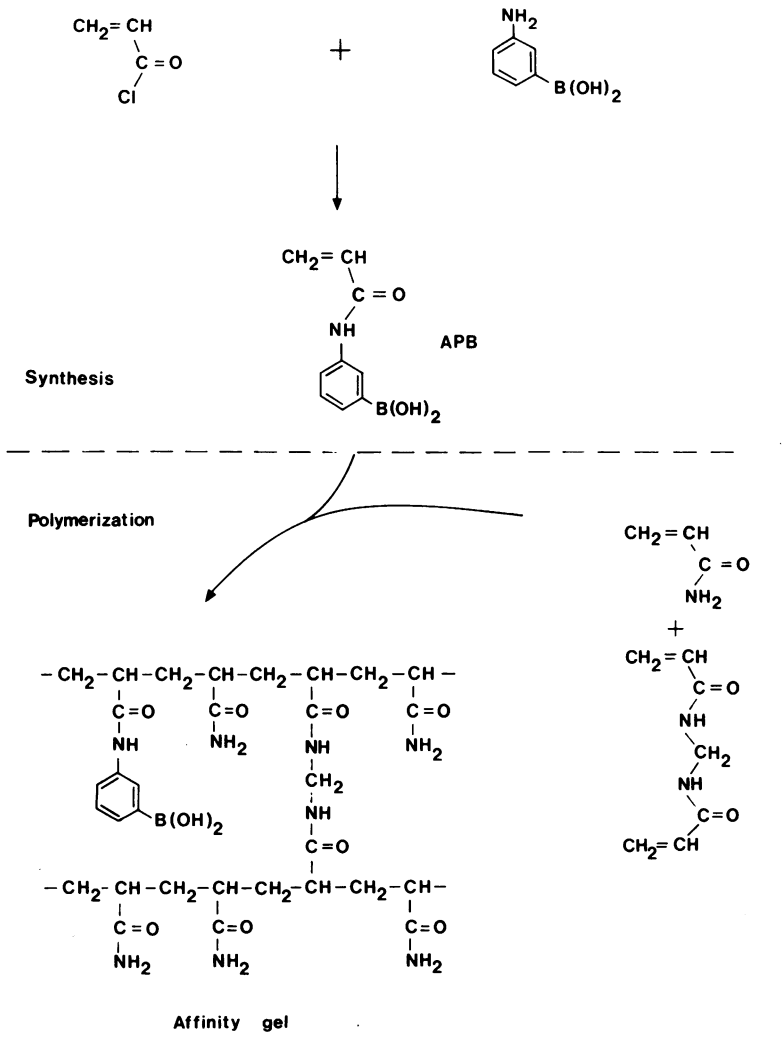
concentration), the following solution was required: 20 mg APB, 4.2 g urea (BRL) dissolved in 1 ml 1 M Tris-AcOH, pH 9.0 (unless specified), 3.26 ml stock acrylamide solution (28.38 g/100 ml acrylamide, 1.62 g/100 ml bisacrylamide) and 0.5 ml 1% ammonium persulphate per 10 ml total volume. Polymerization was initiated with 7 µl Temed.For higher concentration of APB, the amount of stock acrylamide solution was reduced proportionately to maintain a constant total acrylamide concentration. The amount of Bis was not readjusted to take into account the change due to the added APB. The running buffer was 0.1 M Tris-OAc, pH 9.0, unless otherwise indicated.

Elution of radioactive bands was carried out at room temperature overnight by diffusion into 0.3 M NH_4OAc - 20 mM Tris-HCl, pH 7.2. The RNA was recovered by EtOH precipitation, usually in the presence of 10 µg carrier tRNA (*E.coli*) (Boehringer). As in the case of standard gels, non-radioactive RNA was visualized by silver staining, as described (13).

RESULTS

Preparation of *N*-acryloyl-3-aminophenylboronic acid (APB)

3-aminophenylboronic acid was acylated with acryloylchloride in aqueous solution in an analogous manner to that described for other acylations using this reagent (14) (Scheme 1). The resulting *N*-acryloyl-3-aminophenylboronic acid (APB) was stored in a crystalline form in the dark at 4°C and the same handling precautions were taken as is usual for acrylamide. Polymerization of APB in the presence of acrylamide and bisacrylamide (Scheme 1) yielded the affinity gel used in this investigation. The acryloylaminophenylboronate absorbs strongly in the UV region (λ_{max} 254 nm ε^M 13 500 in H_2O) so that RNA in gels containing more than 2 mg/ml APB cannot be visualized using ethidium bromide; methylene blue is a suitable alternative if the RNA is to be eluted. The polyacrylamide gel, modified by the attachment of a boronic acid group, is, at APB concentrations below 5 mg/ml, physically indistinguishable from standard gels. At APB concentrations higher than 10 mg/ml, where the gel becomes rather fragile, the RNA bands no longer retain their usual sharpness, although band separation is such that the distortion does not in-



Scheme 1. Synthesis of *N*-acryloyl-3-aminophenylboronic acid (APB), its copolymerization with acrylamide and the proposed structure of the affinity gel.

fluence the interpretation (see for example Fig. 2). The upper limit for the APB concentration is set by the limited solubility of the monomer in water and is about 15 mg/ml. The capacity of APB-gels for nucleic acids appears to be indistinguishable from standard gels.

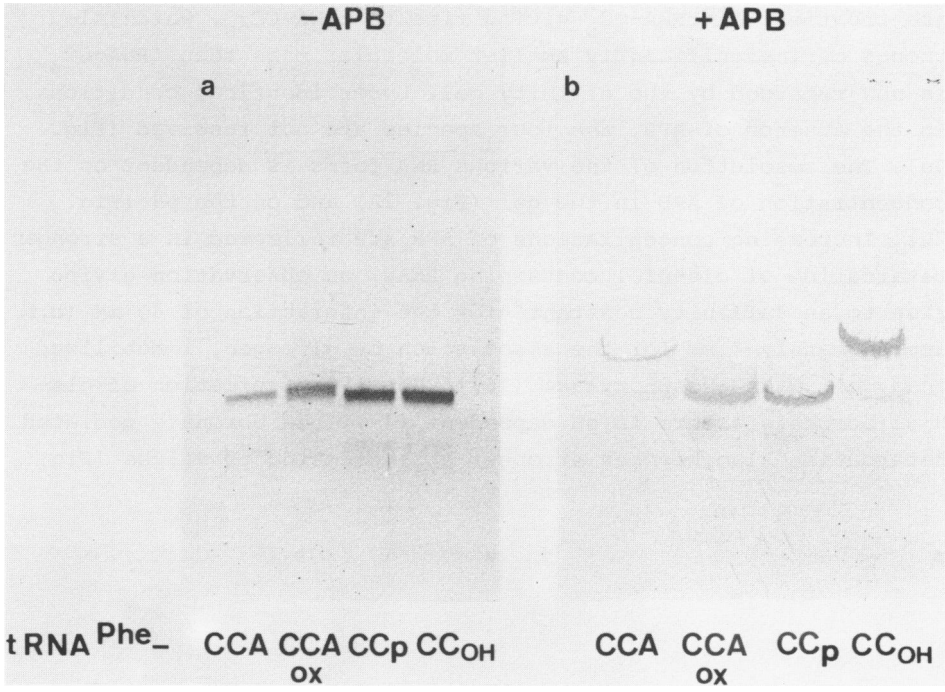
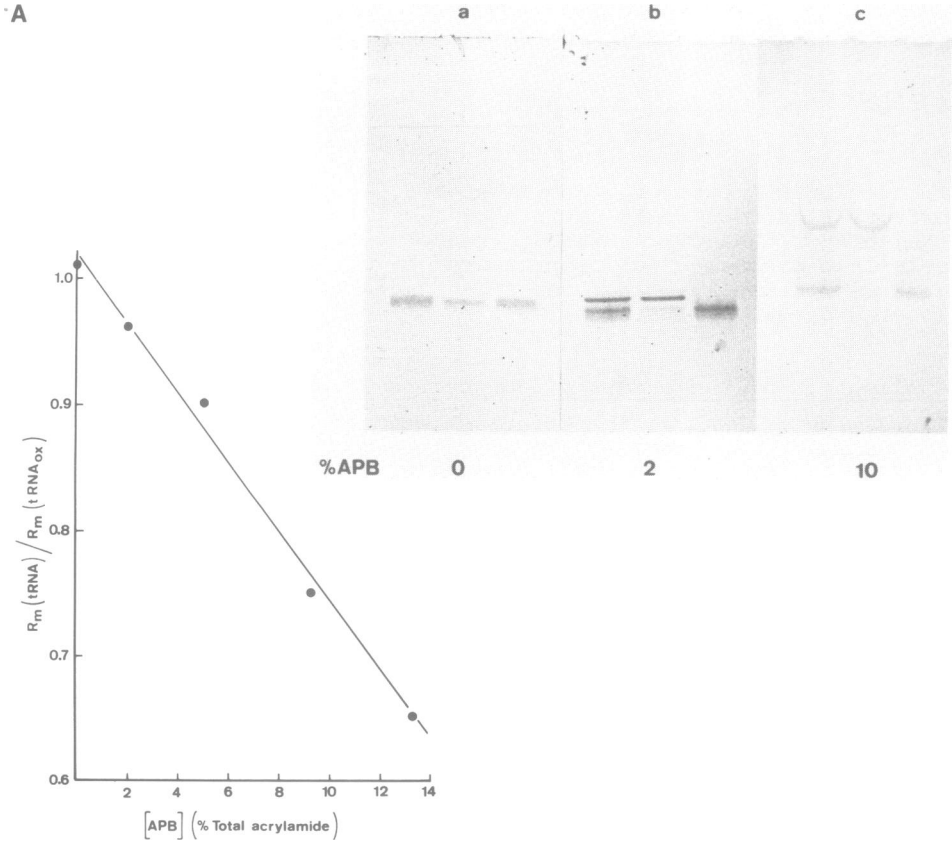


Figure 1. Influence of 3'-terminal modification on the borate effect. Electrophoresis pattern of (left to right) tRNA-CCA, tRNA-CCA_{ox}, tRNA-CC_p, tRNA-CC_{OH} run on 10% acrylamide, 7 M urea gels in the absence (a) or presence (b) of 5% APB. The samples were approximately 20 ng/lane $tRNA^{Phe}$ (yeast) and the bands were located by silver staining (13).

Application of the borate effect to the 3' end of RNA

The 3' end of RNA, as the target of aminoacylation, has been studied intensively (15) and well-defined modifications are available. The stepwise removal of the terminal nucleoside by oxidation-elimination-phosphatase treatment provides a series of intermediates whose behaviour on the boronate gel may be compared with that predicted by their ribose structure (Fig. 1b). Periodate treatment of tRNA-CCA results in the destruction of the ribose ring and a corresponding increase in the mobility of tRNA-CCA_{ox} compared with the native structure. β-elimination of the terminal base of tRNA-CCA_{ox} leads to a shortened tRNA-CC_p with a 3' phosphate terminus and a faster mobility than tRNA-CCA_{ox}. The terminal ribose structure may be restored by phosphat-

ase treatment of tRNA-CC_p giving rise to tRNA-CC_{OH}, which although of insignificantly smaller molecular mass than tRNA-CC_p is now retarded by the affinity gel. Under identical conditions, in the absence of APB, the four species are not resolved (Fig. 1a). The resolution of the various RNA forms is dependent on the concentration of APB in the gel (Fig. 2A) and on the pH (Fig. 2B). Increasing concentrations of APB are reflected in a stronger retardation of cis-diol containing RNAs; an observation giving rise to an "affinity constant" for the interaction of 40 mM (c.f. approximately 1 mM for the association of glycogen, immobilized in a gel, with phosphorylase (16)). Since the formation of cis-diol:boronate esters is pH dependent (17), the boronate mediated retardation also becomes stronger at increasing pH values (Fig.



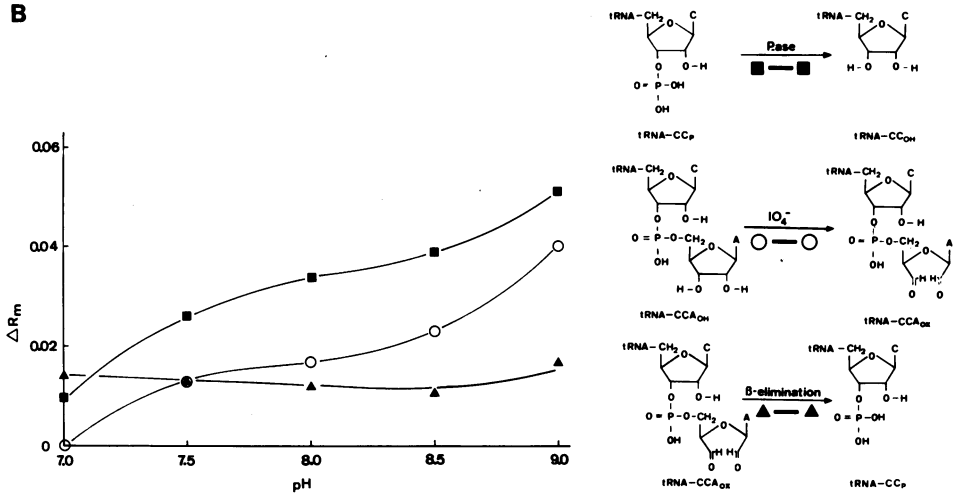


Figure 2. Concentration and pH dependence of the borate effect. R_m is the relative mobility of tRNA-CCA or tRNA-CCA_{ox} with respect to xylene cyanol, which was allowed to migrate to within 2 cm of the end of the 0.5 x 120 x 140 mm gel. (A) The concentration of APB is given as a weight % of the total acrylamide concentration. Inset: a. 0% APB. b. 2% APB. c. 10% APB. Samples were 10 ng tRNA-CCA + 10 ng tRNA-CCA_{ox}, 10 ng tRNA-CCA, 10 ng tRNA-CCA_{ox} (left to right). (B) Samples of $tRNA^{Phe}$ modified as indicated in Fig. 1 were applied to and run on polyacrylamide gels as in (A), containing 2% APB and at the given initial pH. ΔR_m represents the shift in relative mobility between the indicated species. ■—■ tRNA-CC_p → tRNA-CC_{OH}; O—O tRNA-CCA → tRNA-CCA_{ox}; ▲—▲ tRNA-CCA_{ox} → tRNA-CC_p. The 10% acrylamide, 7 M urea gels were run at 5 mA constant current and stained with silver (13).

2B). Confirmation of the implied function of the immobilized boronate group is seen by a comparison of tRNA-CCA_{ox} and tRNA-CC_p (both lacking a free ribose moiety) where the resolution between species is dependent only on size and charge factors. Since, however, the state of ionization of the 3'-terminal phosphate group on tRNA-CC_p (pK 6.2) will not vary greatly over the pH range described, no pH dependence is observed and the resolution may be ascribed to a size difference.

Application of the borate effect to the 5' end of RNA

As indicated above, the pattern of phosphorylation at the 5' end of RNAs offers a number of alternative possibilities. In order to convert these into RNAs amenable to analysis on the affinity gel, two enzymatic reactions were employed.

Figure 3. Detection of 5'-adenylated oligonucleotide 5' monophosphate. A synthetic DNA oligomer, labelled at the 5' end with [^{32}P]monophosphate was adenylated as described in Experimental Procedures in the presence or absence of T4 RNA ligase. It was applied to 0.5 x 120 x 140 mm 10% acrylamide gels containing 7M urea with or without 5% APB, as indicated. The bromphenol blue marker was allowed to migrate to the end of the gel and the bands were located by autoradiography.

5'-monophosphorylated RNAs are characterized as being substrates for T4 RNA ligase, providing that the 5' end is not involved in the secondary structure (18). An obligatory intermediate in this reaction is a 5'-adenylated substrate, of the type A5'pp5'N..., now bearing a free ribose group at the 5' end. A synthetic DNA 30-mer, labelled at the 5' end with ^{32}P, was used as a substrate and, as demonstrated in Fig. 3, the single band of the oligomer is, after ligase treatment, split into two, with about 20% of the counts being retarded. In the absence of APB (Fig. 3), no such fractionation is observed. To confirm the nature of the

Table 1. Phosphatase Sensitivity of RNA Ligase-Treated Isolated Oligomers.

Sample Preincubation	+ligase/upper band		+ligase/lower band		-ligase	
	+NPPase	-NPPase	+NPPase	-NPPase	+NPPase	-NPPase
	counts/min					
	689	653	3574	3381	3219	3060
+Phosphatase	65	713	380	98	267	73

The material eluted from the gel was preincubated with 20 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.5 mg/ml salmon sperm DNA ± 0.12 µg nucleotide pyrophosphatase (NPPase) (*Crotalus atrox*, Sigma) in a total volume of 50 µl, 15 min at 37°C. 20 µl were withdrawn and tested for CCl_3COOH precipitable counts. 1 µg (1 µl) bacterial alkaline phosphatase was added to each of the remaining samples and the reaction continued for 30 min at 37°C. 20 µl aliquots were analyzed for CCl_3COOH precipitable counts.

boronate-retarded band, the material from both sections of the gel was eluted and tested for sensitivity towards alkaline phosphatase (Table 1). Phosphatase digestion of the upper-band material could only be achieved after pretreatment with nucleotide pyrophosphatase, indicating the presence of the adenylated intermediate (19). 5' di- or triphosphate-terminating RNAs are substrates for the capping enzyme, guanylyltransferase (20), which, *in vitro*, brings about the addition of a 5'-linked GMP to the terminus. As in the case of RNA ligase, the newly created free ribose becomes available for interaction with the boronate gel. Fig. 4a demonstrates the behaviour of a 5'-capped primary transcript (see Experimental Procedures) compared with the uncapped species (lanes 1 cf. 2). A substantial proportion of the RNA subjected to capping is retarded by the gel, giving a separation even after a short migration distance. To test the borate effect due solely to the interaction of the 5' cap without interference from the free 3' end, the transcript was periodate oxidized prior to capping. By comparing lanes 3 and 4 (Fig. 4a) with lanes 1 and 2 it is seen that the contribution of the 3' ribose and the 5' ribose to the borate effect complement each other and give rise to an increased retardation. It is, however, noticeable that the effect due to the 5' cap ribose appears weaker than the

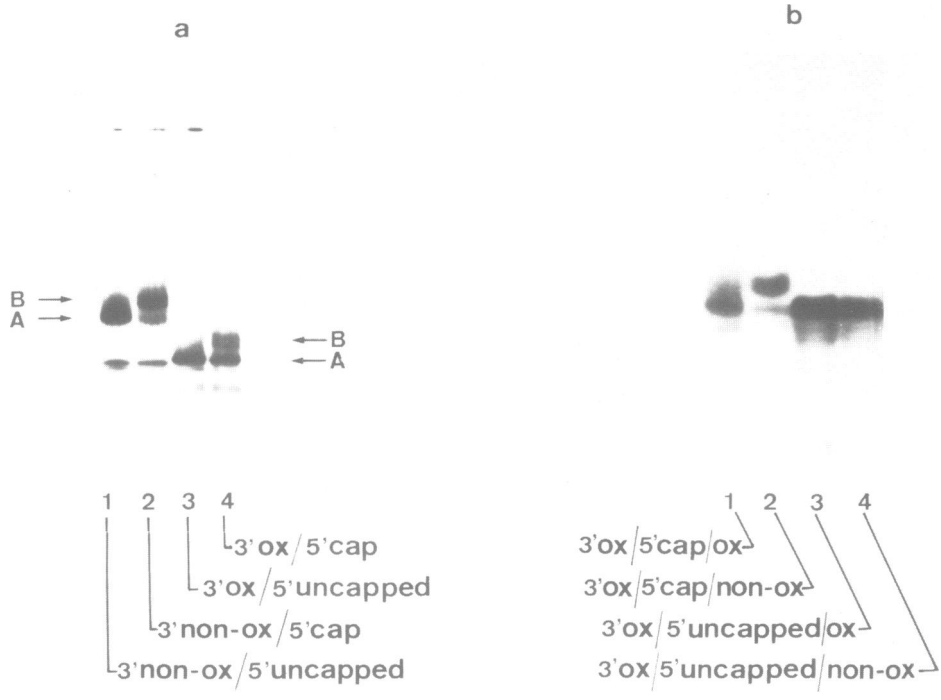


Figure 4. Detection of a 5'-capped transcript. A 340 nucleotide *in vitro* transcript (see Experimental Procedures) was subjected to capping with non-radioactive GTP in the presence of guanylyltransferase, or was used in controls for this reaction. a) Electrophoresis of the products of the capping reaction on a 10% acrylamide, 7 M urea, 5% APB gel. Samples were as indicated on the figure. Arrows at A indicate uncapped RNA, B marks bands retarded by the gel as a result of capping. Migration distance of band A, lanes 3 and 4 was 7.5 cm. b) Band A from lane 3 and band B from lane 4 in part a) were eluted and half of each sample was periodate oxidized. The products were analyzed on a 10% acrylamide, 7 M urea, 5% APB gel, as described.

3' interaction. This may be due to the effect of a more ordered structure at the 5' end being shielded from interaction with the boronic acid. It should be pointed out that the apparently incomplete conversion of transcripts to capped derivatives is probably due to the sensitivity of the 5'-terminal triphosphate to the isolation conditions in these experiments. To confirm that the 5' cap itself contributed to the retardation of the RNA by the APB gel, band A from lane 3 (3' oxidized/uncapped) and band B from lane 4 (3' oxidized/capped) (Fig. 4a) were eluted and half

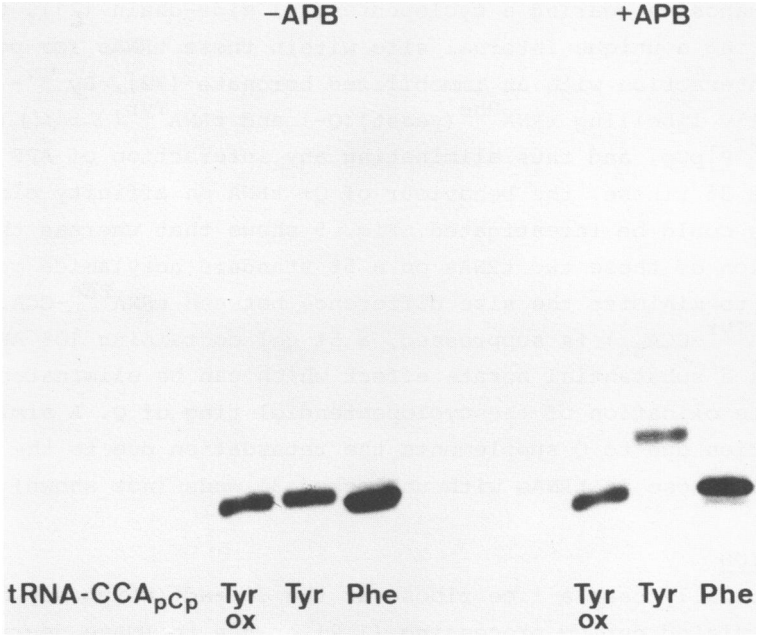


Figure 5. Detection of the modified base Q in tRNA. tRNA^{Phe}(yeast) and tRNA^{Tyr}(*E.coli*) were labelled at the 3' end with [^{32}P]pCp using T4 RNA ligase (18). An aliquot of the labelled tRNA^{Tyr} was subjected to periodate oxidation. Samples of each of the tRNA species were analyzed on a 5% acrylamide, 7 M urea gel in 0.1 M Tris-OAc with or without 10% APB as a copolymeric component, as indicated. The bromphenol blue marker was allowed to run off the bottom of the 0.5 x 120 x 140 mm gel.

of the material was subjected to another round of periodate oxidation. Fig. 4b shows that the ribose residue of the 5' cap and consequently the interaction with the gel is destroyed by this treatment so that the RNA (3' oxidized/capped/5' oxidized) (lane 1) now migrates faster than the 3' oxidized/capped species (lane 2) to a position equivalent to the 3' oxidized/uncapped controls (lanes 3 and 4). The pattern depicted in Fig. 4 is clear evidence of the "cappability" of the RNA used as a model and that the boronate affinity electrophoresis gels are suitable tools for monitoring the termini of primary transcripts.

Application of the borate effect to internal nucleotides: the Q base

The modified base Q occurs at position 34 in several of those tRNAs which read XA(U/C) codons (5). Q is a derivative of 7-

deaza-guanosine bearing a cyclopentendiol side-chain (21), thereby offering a unique internal site within these tRNAs for possible interaction with an immobilized boronate (22). By 3'-terminally labelling $tRNA^{Phe}$(yeast)(Q-) and $tRNA^{Tyr}$(*E.coli*) (Q+) with [^{32}P]pCp, and thus eliminating any interaction of APB with the 3' ribose, the behaviour of Q+ tRNA on affinity electrophoresis could be investigated. Fig. 5 shows that whereas the resolution of these two tRNAs on a 5% standard acrylamide gel (chosen to minimize the size difference between $tRNA^{Phe}$-CCA_{76} and $tRNA^{Tyr}$-CCA_{85}) is suppressed, a 5% gel containing 10% APB produces a substantial borate effect which can be eliminated by periodate oxidation of the cyclopentendiol ring of Q. A similar retardation due to Q supplements the retardation due to the 3'-terminal ribose in tRNAs with unblocked 3' ends (not shown).

DISCUSSION

RNAs typically bear a free ribose at the 3' end (which may be phosphorylated during processing (1,2) or due to RNAse degradation (3)) but may also contain cis-diol groups internally (e.g. the hypermodified base Q of some tRNAs (5)) or additional ribonucleotides due to maturation processes (e.g. 5'-terminal G of the mRNA cap structure). A reliable, non-destructive analytical method specific for free ribose functions would facilitate investigations of the state of modification of these sites and would provide evidence of pathways leading to their formation. One cis-diol specific interaction which has been well-characterized is the formation of boronic acid: ribose complexes which have been exploited for many years in paper electrophoresis (23) and in affinity chromatographic (17,24) fractionation of ribonucleosides and short oligomers. However, attempts to use boronate resins for the analysis of longer RNAs have met with mixed success or low reproducibility, so that a number of improvements have been put forward (22,25,26). These advances have been mainly aimed at providing a system for the separation of aminoacylated tRNAs from uncharged tRNA. Affinity chromatography of this type may well be the method of choice for the particular problem where the instability of the aminoacyl link is compatible with the rapid manipulation provided by the column methods. For

other applications, including those for which chromatography has previously been attempted, as well as potential novel procedures, the improved resolution of gel electrophoresis over chromatography would seem to warrant the development of affinity electrophoresis (7,8), combining the property of boronates with the well-known advantages of electrophoresis.

To this end, we have designed an affinity gel electrophoretic system, using *N*-acryloyl-3-aminophenylboronic acid as a copolymeric component and have applied it to the isolation and non-destructive analysis of such RNAs whose structure, at a variety of sites, permit interaction with the gel matrix.

It has been shown by manipulating the 3' terminus of tRNA that the affinity of the gel for RNA is influenced exclusively by the presence or absence of a free ribose function. This was not only the case with periodate mediated destruction of the diol structure but also with phosphorylation/dephosphorylation of the 3' terminus (Fig. 1) so that one may advantageously establish the nature of 3' phosphorylation for any given RNA fragment without the more usual and drastic nucleolytic analysis.

The state of the 5' end in the context of RNA transcripts often provides vital information in determining the nature and function of an RNA species, the mechanism leading to its formation or the fate of its precursor. However, with the techniques of S_1-mapping and reverse transcriptase mapping commonly used for locating the position of the terminal nucleotide of RNAs (27,28) it is not possible to identify the state of RNA termini. Specifically, one must distinguish the characteristic 5'pppN... of a primary transcript from the processed 5'pN... or 5'HON... Whereas one may simply phosphorylate a free 5'OH by standard procedures to establish its presence, a differentiation between 5'pN... and 5'pppN... has required terminal nucleotide analysis. T4 RNA ligase acts specifically on 5' monophosphate-bearing RNAs to give, in the presence of ATP, an adenylated intermediate (19). This intermediate has previously only been characterized as being present in enzymatic digests. We have shown that the ribose ring of the terminal A (i.e. in the form A5'pp5'N...) interacts with the boronate affinity gel, resulting in a retardation of this species. In this way, not only is the ligase reaction

an appropriate assay for the presence of a 5' monophosphate but the electrophoretic system provides a means of isolating the adenylated RNA (e.g. as a means of recovering 5'-terminal fragment from a partial RNAse digest as in the recent example of the enzymatic replacement of the anticodon region of tRNAs (29)). One should mention, that such adenylation reactions are not restricted to T4 RNA ligase but have also been implicated in RNA splicing mechanisms (1) and in the post-transcriptional 5'-guanylation of eukaryotic $tRNA^{His}$ species (30). An extension of the boronate gels to the assay of such pathways would appear to be appropriate.

In order to examine the application of boronate gels to the differentiation between capped (or cappable) RNAs, i.e. primary transcripts, and mature 5' monophosphate-bearing RNAs, we made use of the ribose ring of the 5'-terminal G, introduced through capping *in vitro*. The capping reaction, specific for 5' di- or triphosphates, has been used previously in defining transcriptional start sites (12,20) but its sensitivity is highly dependent on the amount of precursor available for capping. Furthermore, in view of the relatively high K_m of guanylytransferase for GTP (15 µM) (31), it is unlikely that complete incorporation of high-specific-activity GTP is ever attained. If, on the other hand, the RNA is itself radioactively labelled by, e.g. transcription *in vitro* or by 3'-terminal labelling, capping with unlabelled GTP at enzyme-saturating concentrations is possible, provided that a method for separating capped from uncapped RNA is available.

Thus, a single ribose residue is sufficient even in chain lengths of over 300 nucleotides for affinity fractionation. Consequently, and considering the limitation in chain lengths set by the nature of polyacrylamide gels in general, the separation of capped from uncapped RNA by a one step procedure has become feasible. Previously, this has only been possible by affinity chromatography of RNA capped *in vitro* using GTP(αS) (32) on a mercurated resin; boronate affinity electrophoresis permits direct fractionation of RNA capped *in vivo*. The termini of RNA have been extensively characterized with respect to their interaction with the boronate affinity gel.

Of considerable interest, however, is also the modified base Q occurring in certain tRNAs (5). Queuosine appears to play a role in the translational activity of tRNA (33) and to have developmental/differentiation (6) implications. Group specific separation of Q-containing tRNAs has been demonstrated previously on boronate affinity columns (22). The affinity electrophoretic system presented here possesses similar properties with the added potential of being able to incorporate the affinity gel electrophoretic step into a two-dimensional electrophoretic procedure in order to resolve the tRNA mixture into its individual components without necessitating prefractionation. Indeed, the identification of Q+ tRNAs in a given bulk tRNA two-dimensional pattern could contribute towards the understanding of the function of Q in development, differentiation and distribution in various cell types.
We have shown in this study that the principles previously established for boronate affinity chromatographic media may be extended with additional applications to affinity electrophoresis. The development of the electrophoretic gels described in this report offers not only a further analytical technique in the study of RNA structure but also the possibility of isolating RNAs bearing well-defined terminal or internal nucleotides on a preparative and quantitative scale.

ACKNOWLEDGEMENTS

We would like to thank Dr. G. Feix for critical comments on the manuscript. We are grateful to Mrs. E. Schiefermayr for assistance in the preparation of the figures. This work was supported by the *Deutsche Forschungsgemeinschaft (Schwerpunkt Molekularbiologie der Pflanzen)* and by the *Fond der Chemischen Industrie.*

REFERENCES

1. Hopper, A.K. (1984) in Processing of RNA, Apirion, D., Ed., pp. 91-117, CRC Press, Boca Raton, Florida.
2. Flint, S.J. (1984) in Processing of RNA, Apirion, D., Ed., pp. 151-179, CRC Press, Boca Raton, Florida.
3. Blackburn, P. and Moore, S. (1982) in The Enzymes, Boyer, P.D., Ed., Vol. XV, pp. 317-433, Academic Press, New York, London.
4. Rhoads, R.E. (1985) Prog. Mol. Subcell. Biol. 9, 105-155.

5. Nishimura, S. (1978) in Transfer RNA, Altman, S., Ed., pp. 168-195, The MIT Press, Cambridge, Mass. and London.
6. Nishimura, S. (1983) Prog. Nucl. Acids Res. Mol. Biol. 28, 49-80.
7. Hořejší, V. (1981) Anal. Biochem. 112, 1-8.
8. Hořejší, V. (1984) Methods Enzymol. 104, 275-281.
9. Igloi, G.L., von der Haar, F. and Cramer, F. (1979) Methods Enzymol. 59, 282-291.
10. Lau, C.K. and Fujitaki, J.M. (1981) Anal. Biochem. 110, 144-145.
11. Tal, J., Deutscher, M.P. and Littauer, U.Z. (1972) Eur. J. Biochem. 28, 478-491.
12. Strittmatter, G., Gozdzicka-Jozefiak, A. and Kössel, H. (1985) EMBO J. 4, 599-604.
13. Igloi, G.L. (1983) Anal. Biochem. 134, 184-188.
14. Whiteside, G.M., Lamotte, A., Adalsteinsson, O. and Cotton, C.K. (1976) Methods Enzymol. 44, 887-897.
15. Sprinzl, M. and Cramer, F. (1979) Prog. Nucl. Acids Res. Mol. Biol. 22, 2-69.
16. Takeo, K. and Nakamura, S. (1972) Arch. Biochem. Biophys. 153, 1-7.
17. Weith, H.L., Wiebers, J.L. and Gilham, P.T. (1970) Biochemistry 9, 4396-4401.
18. Bruce, A.G. and Uhlenbeck, O.C. (1978) Nucl. Acids Res. 5, 3665-3677.
19. Sugino, A., Snopek, T.J. and Cozzarelli, N. (1977) J. Biol. Chem. 252, 1732-1738.
20. Levens, D., Ticho, B., Ackerman, E. and Rabinowitz, M. (1981) J. Biol. Chem. 256, 5226-5232.
21. Kasai, H., Ohashi, Z., Harada, F., Nishimura, S., Oppenheimer, N.J., Crain, P.F., Liehr, J.G., von Minden, D.L. and McCloskey, J.A. (1975) Biochemistry 14, 4198-4208.
22. McCutchan, T.F., Gilham, P.T. and Söll, D. (1975) Nucl. Acids Res. 2, 853-864.
23. Ferrier, R.J. (1978) Adv. Carb. Chem. 35, 31-80.
24. Schott, H., Rudloff, E., Schmidt, P., Roychoudhury, R. and Kössel, H. (1973) Biochemistry 12, 932-938.
25. Singhal, R.P., Bajaj, R.K., Buess, C.M., Smoll, D.B. and Vakharia, V.N. (1980) Anal. Biochem. 109, 1-11.
26. Johnson, B.J.B. (1981) Biochemistry 20, 6103-6108.
27. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 721-732.
28. Efstratiadis, A., Kafatos, F.C., Maxam, A.M. and Maniatis, T. (1976) Cell 7, 279-288.
29. Wittenberg, W.L. and Uhlenbeck, O.C. (1985) Biochemistry 24, 2705-2712.
30. Cooley, L., Appel, B. and Söll, D. (1982) Proc. Natl Acad. Sci. USA 79, 6475-6479.
31. Monroy, G., Spencer, E. and Hurwitz, J. (1978) J. Biol. Chem. 253, 4490-4498.
32. Smith, M.M., Reeve, A.B. and Huang, R.C.C. (1978) Biochemistry 17, 493-500.
33. Beier, H., Barciszewska, M. and Sickinger, H.-D. (1984) EMBO J. 3, 1091-1096.