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Nucleotide Sequence That Binds Primer for DNA Synthesis to the Avian Sarcoma Virus Genome

BARBARA CORDELL,* EDWARD STAVNEZER, ROLAND FRIEDRICH, J. MICHAEL BISHOP, AND HOWARD M. GOODMAN

Department of Microbiology and Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143*

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Initiation of transcription from the genome of avian sarcoma virus by RNA-directed DNA polymerase in vitro requires $tRNA^{trp}$ as a primer. The tRNA is bound to the viral genome by a sequence of 16 contiguous nucleotides (U-C-A-C-G-U-C-G-G-G-G-U-C-A-C-Cp), beginning with the penultimate base at the 3′ terminus of the primer and extending through the acceptor stem into loop IV of the tRNA. Consequently, the native conformation of the tRNA must be disrupted by the binding of primer to the viral genome. The binding sequence does not include two adjacent residues of pseudouridine in loop IV, which distinguish the primer from many other tRNAs, and the 3′ terminal adenosine of primer may also be excluded from base pairing with the viral genome.

The genome of avian sarcoma virus (ASV) is composed of two identical subunits, each containing approximately 10,000 nucleotides (3, 4, 19). Bound to each subunit is a 4S RNA primer, which is required for the initiation of DNA synthesis in vitro by the virion RNA-directed DNA polymerase (9, 11, 13, 26). The 4S primer has been shown to be identical to the cellular $tRNA^{trp}$ (17; our unpublished data). Removal of the primer from the high-molecular-weight subunit results in loss of template activity for RNA-directed DNA polymerase (7, 9).

The specificity and stability that characterize the binding of primer to viral genome are important elements in the initiation of DNA synthesis (23). To determine the nucleotide sequence that mediates this binding, we exploited the fact that primer can be reassociated with high-molecular-weight subunits of the ASV genome to reconstruct a complex structurally and functionally similar to the native complex between primer and viral genome: the temperatures required to denature the native and reconstructed primer-template complexes are identical (9, 23); native and reconstructed complexes have roughly equivalent template activites (12, 23); and the same initial sequence of deoxynucleotides (dA-dA-dT-dG-dA-dA-dG-dC) is found in transcripts from native and reconstructed complexes (24; R. Friedrich, unpublished data). We annealed ^{32}P-labeled primer to unlabeled high-molecular-weight subunits of ASV RNA, isolated a duplex resistant to hydrolysis by RNase, and analyzed the nucleotide sequence of the ^{32}P-labeled constituent of the duplex. Our results conform to those reported recently by Eiden et al. (10) and, in addition, define the exact 3′ and 5′ termini of the binding sequence.

MATERIALS AND METHODS

Materials. The sources of most materials have been described previously (9, 13). [^{32}P]orthophosphate was purchased from International Chemical and Nuclear Corp. Agarose (A15) was purchased form Bio-Rad Laboratories. Enzymes were obtained from the following sources: RNase T_1 (EC 3.1.4.8) for fingerprint analysis, RNase T_2 (EC 3.1.4.23), and RNase U_2 from Calbiochem; RNase A (EC 3.1.4.22), RNase T_1 (EC 3.1.4.8) for preparation of RNase-resistant duplexes, bacterial alkaline phosphatase (EC 3.1.3.1), and snake venom phosphodiesterase (EC 3.1.4.1) from Worthington Biochemicals Corp. Purified S_1 nuclease was a gift from T. Ando, Tokyo, Japan.

Propagation and purification of virus. The propagation of the B77 (subgroup C) strain of ASV was carried out as described previously (5, 9). Labeling of virus with [^{32}P]orthophosphate was accomplished by incubating infected cells in phosphate-free medium containing 2 mCi of radioisotope per ml, harvesting, and replenishing with fresh medium containing label twice per day over a 3-day period. RNA with a specific activity of about 20,000 counts/min per ng was obtained. The Prague strain of ASV (subgroup C) was obtained as concentrated suspensions from University Laboratories, Highland Park, N.J., under the auspices of the Office of Program Resources and Logistics, National Cancer Institute.

Isolation of viral RNAs. RNA was extracted from purified virus as described previously (5) and separated into 70S and low-molecular-weight fractions by rate-zonal centrifugation (5). The 70S RNA was

denatured by heating at 80 C for 2 to 4 min at concentrations no greater than 250 μg/ml in 0.01 M EDTA-0.02 M Tris-hydrochloride, pH 7.4. High-molecular-weight subunits were then separated from the low-molecular-weight RNAs by gel filtration through agarose (A15) as described below.

Purification of tRNA primer from ASV. The primer was separated from other low-molecular-weight RNAs found in the virion and obtained in pure form, using two-dimensional electrophoresis in slab gels (12 by 17 cm); the first dimension was in 10% polyacrylamide for 3.5 h at 350 V, and the second was in 20% polyacrylamide for 16 h at 350 V. This procedure has been described in detail (9, 18). Primer labeled with ^{32}P was located in the slab gel be autoradiography, excised, and eluted by shaking the crushed gel in 0.3 M NaCl at room temperature for 3 to 4 h. The eluate, containing more than 90% of the RNA, was passed through a glass-fiber filter (Reeve-Angel 93A AH), and the RNA filtrate was precipitated with ethanol.

Cleavage of tRNA primer with nuclease S_1. ASV primer was treated with purified S_1 nuclease according to published procedures (16). ^{32}P-labeled tRNA primer was incubated with S_1 (100 μg/ml) for 2 h at room temperature in 0.3 M NaCl-0.03 M NaOAc-3 mM $ZnCl_2$, pH 4.5, in the presence of 20 μg of yeast 4S carrier RNA. The resultant 3′ and 5′ halves of the tRNA primer were separated by electrophoresis in slab gels of 20% acrylamide (12 by 17 cm) for 4 h at 400 V. RNA fragments were eluted from gel slices as described above, and their position in the tRNA was confirmed by nucleotide sequence analysis (21).

Annealing of RNA. Primer and subunit RNAs were annealed in 0.6 M NaCl-0.01 M EDTA-0.02 M Tris-hydrochloride, pH 7.4, at 74 C to 20 times the $C_rt_{1/2}$ of the reaction (23).

Isolation of RNase-resistant duplex RNAs. The following conditions were found to hydrolyze greater than 95% of a single-stranded RNA (ASV, 35S [^{32}P]-RNA and less than 5% of a duplex RNA (poliovirus, ^3H-replicative form): 5 U of RNase T_1 (Worthington) per ml plus 50 μg of pancreatic RNase per ml in 0.5 M NaCl-0.01 M EDTA-0.02 M Tris-hydrochloride, pH 7.4, for 1 h at 37 C at an RNA concentration of 500 μg/ml (by supplementation with yeast 4S carrier); and 8 U of RNase T_2 per ml in 0.5 M NaCl-1 mM EDTA-0.01 M NaOAc, pH 4.5, for 1 h at 37 C at an RNA concentration of 500 μg/ml. The digestion mixes were diluted fourfold with 0.01 M EDTA-0.02 M Tris-hydrochloride, pH 7.4, extracted twice with phenol, treated with 0.05% diethylpyrocarbonate for 30 s, and then ethanol precipitated.

Gel filtration through agarose. Columns (40 by 0.9 cm) of agarose (Bio-Rad, A15m) were used at room temperature. Filtration was carried out in 0.3 M NaCl-0.01 M EDTA-0.02 M Tris-hydrochloride-0.5% (wt/vol) sodium dodecyl sulfate as described previously (23).

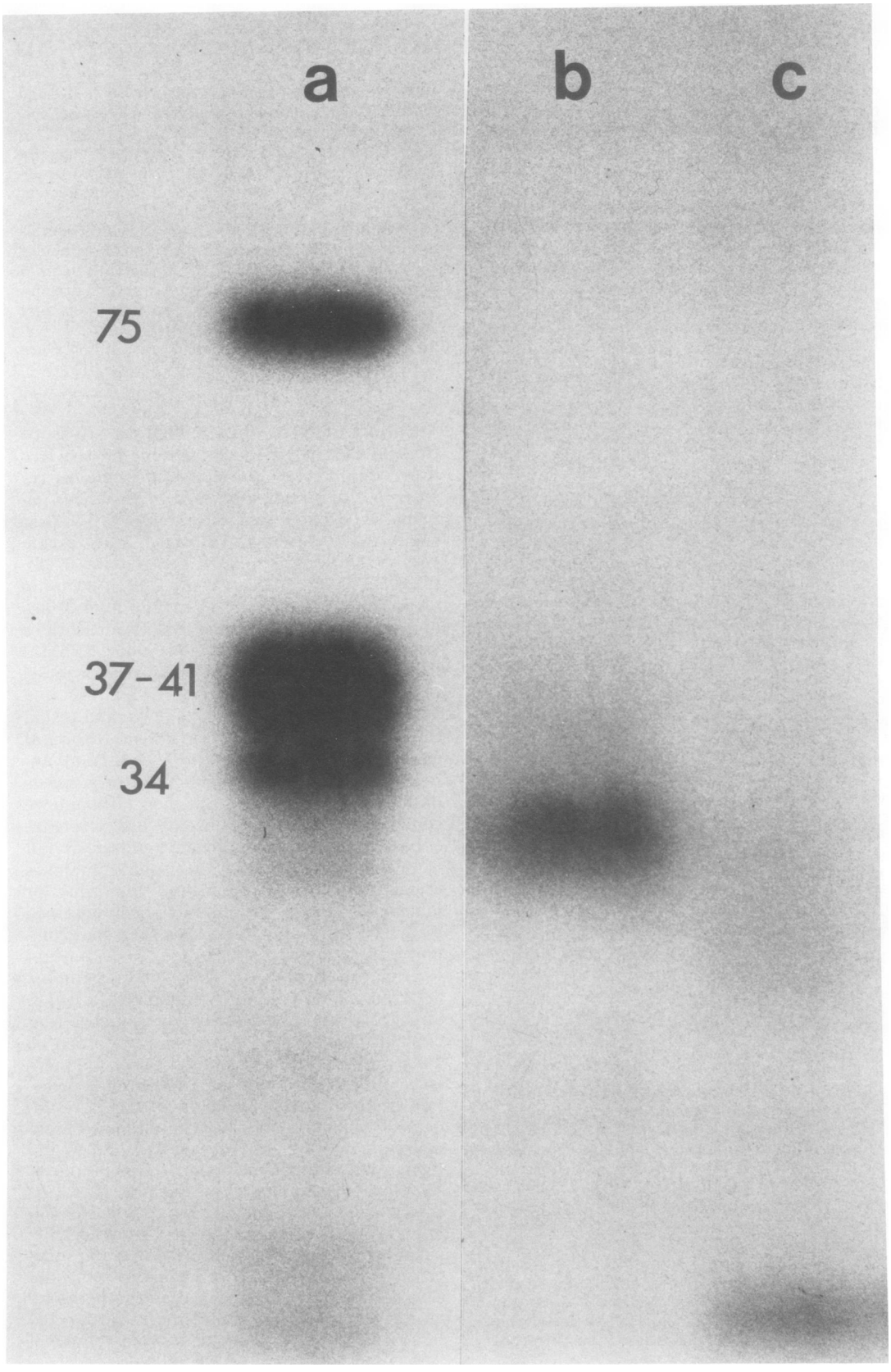
Nucleotide sequence analysis. ^{32}P-labeled tRNA, tRNA primer, and primer segments were hydrolzyed with T_1 RNase (Calbiochem), and the resulting oligonucleotides were separated by two-dimensional paper electrophoresis as described by Sanger et al. (21). Individual oligonucleotides from fingerprints were redigested separately with RNases A, T_2, and U_2, and the products were analyzed by electrophoresis on DEAE paper at pH 3.5 (RNase A products), on 3MM paper at pH 3.5 (RNase T_2 products), and on DEAE paper at pH 1.9 (RNase U_2 products) as described by Adams et al. (1), Barrell (2), and Brownlee (6).

β-Elimination. β-Elimination was performed as described by Fraenkel-Conrat and Steinschneider (14). Briefly, the sample was oxidized with 0.2 mM periodate for 1 h at 0 C in the dark and then precipitated twice with isopropanol. The RNA was next treated with 0.3 M aniline-0.01 M NaOAc, pH 5.0, for 3 h at room temperature and precipitated twice with isopropanol.

RESULTS

Localization of the nucleotide sequence that binds $tRNA^{trp}$ to the genome of ASV. To provisionally locate the nucleotide sequence responsible for binding primer to the viral genome, we exploited the recent observation that single-strand-specific nucleases can divide tRNA molecules approximately in half (16, 22). For example, S_1 nuclease cleaves $tRNA^{trp}$ in the anticodon and removes up to five nucleotides and a 5′ terminal fragment of 34 nucleotides (16; our unpublished data). We cleaved ^{32}P-labeled ASV primer with S_1 nuclease, isolated the resulting fragments by electrophoresis in a polyacrylamide gel (Fig. 1, lane a), and tested the ability of the fragments to anneal with viral genome (Table 1). Only the 3′ fragment annealed; hence, the principal binding sequence is located in the 3′ half of the primer, although we cannot exclude the existence of short stretches of base pairing between genome and the 5′ half of primer which do not form stable duplexes under our relatively stringent conditions for annealing. Similar results have been obtained by J. Dahlberg and co-workers (personal communication).

Preparation of an RNase-resistant duplex between primer and ASV genome. To facilitate direct analysis of the nucleotide sequence that binds primer to the ASV genome, we prepared an RNase-resistant duplex between radiolabeled $tRNA^{trp}$ and the complementary nucleotide sequence in unlabeled viral RNA. We annealed ^{32}P-labeled primer with unlabeled high-molecular-weight subunits of ASV RNA and then separated the reassociated primer-subunit complex from unbound primer by filtration through agarose columns (Fig. 2). These columns retard the passage of low-molecular-weight RNAs (either unannealed or preincubated under annealing conditions in the absence of 35S RNA [23]), whereas the high-molecular-weight subunits and the reconstructed ^{32}P-4S-35S complex elute in the void volume of


a
b
c
75
37-41
34

the column. RNA was precipitated from the void volume and subjected to hydrolysis with T_1 plus pancreatic RNases under conditions specific for the degradation of single-stranded RNA.

Approximately 15 to 20% of the primer RNA is contained in an RNase-resistant duplex when hybridized to genome subunits (Table 2). Native primer or primer annealed with yeast RNA is only 0.6% RNase resistant under identical hydrolysis conditions (Table 2). This suggests that roughly 12 to 15 nucleotides of the 75 bases comprising primer participate in duplex formation with the viral genome. The isolated duplex itself is completely RNase resistant when retreated with nucleases (Table 2).

Size and homogeneity of the duplex between primer and ASV genome. The nuclease-resistant duplex between primer and ASV genome migrated as a single species when analyzed by electrophoresis in polyacrylamide (Fig. 1, lane b). Denaturation of the duplex produced a single, more rapidly migrating form (Fig. 1, lane c), which was completely sensitive to hydrolysis by RNase after elution from the gel (data not shown). Since both native and denatured duplexes were homogeneous in size, we concluded that the duplex was formed with an uninterrupted sequence of nucleotides in the 3′ portion of the primer.

We could not properly evaluate the size of the

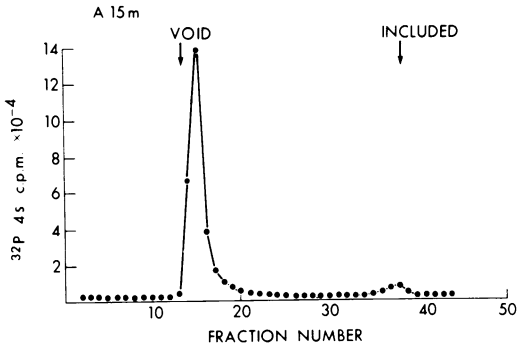


FIG. 2. *Separation of annealed primer-subunit complex from unannealed primer by gel filtration on agarose A15. Preparation and annealing of [32P]primer and ASV high-molecular-weight subunits are described in Materials and Methods. Annealed and unannealed [32P]primer were fractionated by passage through agarose (A15) at a rate of 0.4 ml/min. Radioactivity in individual fractions was measured by Cerenkov radiation in a liquid scintillation counter. The void volume and the included 4S RNA are indicated.*

TABLE 1. *Annealing of ASV subunits and primer halves produced by cleavage with S_1 nuclease*[a]

Primer	Counts/min		% Annealed
	Total	Annealed	
Untreated native primer	382	334	87.4
3′ Half of primer from S_1 treatment	193	159	82.4
5′ Half of primer from S_1 treatment	226	22	9.7

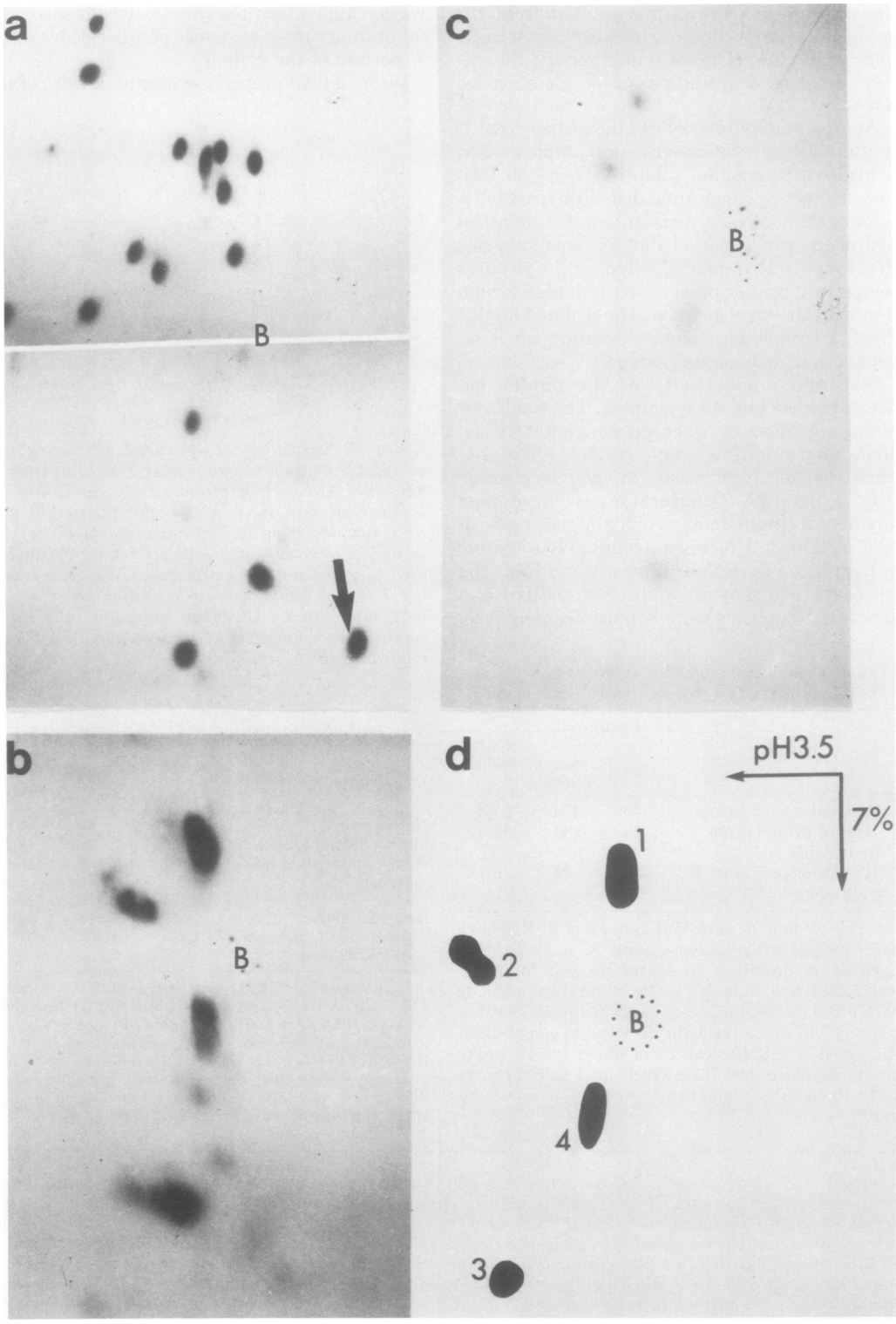
[a] The 3′ and 5′ terminal halves of [32P]primer were produced by cleavage with S_1 nuclease and purified as described in Materials and Methods. Each RNA was annealed under standard conditions with a 100-fold molar excess of ASV subunits (500 to 1,000 ng) to drive the hybridization to completion. Annealed [32P]RNA was determined by an assay previously described (23), which uses electrophoresis in 10% polyacrylamide cylindrical gels (6 mA/gel per h).

TABLE 2. *RNase resistance of tRNA primer before and after annealing with ASV subunits*[a]

Determination	Counts/min		% Resistant
	− RNase	+ RNase	
Native primer	4,500	28	0.6
Mock annealed primer	4,900	28	0.6
Primer annealed to ASV genome RNA	903	118	13
	18,200	3,542	19
	273	49	18
Isolated duplex (prepared with T_1 and pancreatic RNases)	561	516	92
	1,325	1,256	94

[a] Samples of [32P]RNAs were dissolved in 0.5 M NaCl-0.01 M EDTA-0.02 M Tris-hydrochloride, pH 7.4, brought to 500 μg of RNA per ml with yeast RNA, divided in half, and incubated in the presence (+) or absence (−) of 5 to 10 U of RNase T_1 and 50 μg of pancreatic RNase per ml at 37 C for 1 h. Samples were then assayed for acid-insoluble counts. Backgrounds of 4 to 10 counts/min (depending on the individual experiment) have been subtracted.

FIG. 1. *Gel electrophoresis of the primer-subunit RNase-resistant duplex. [32P]primer-subunit duplex produced by digestion with RNase T_1 and pancreatic RNase was suspended in water. A part of the sample was treated with 90% dimethyl sulfoxide at 80 C for 3 min; the remainder was held at 0 C. The samples were ethanol precipitated, resuspended in 25 μl of 0.01 M EDTA, 0.02 M Tris-hydrochloride, pH 7.4, containing 15% sucrose and loaded on a 20% polyacrylamide slab gel (12 by 17 cm). Electrophoresis was carried out for 4 h at 400 V and 16 C. An S_1 nuclease digest of 32P-labeled primer was obtained as described in Materials and Methods and run concomitantly as marker RNAs. RNA species were located in the gel by autoradiography. Lane a displays primer (75 nucleotides) and the "halves" of primer (34 and 37 to 41 nucleotides) obtained by cleavage with S_1 nuclease. Lane b is primer-subunit duplex. Lane c is the denatured [32P]primer-subunit duplex.*


a
B
c
B
b
B
d
pH3.5
7%
1
2
B
4
3

duplex from its electrophoretic mobility because we had no duplex standards of suitable molecular weights. However, we compared the mobilities of native and denatured duplex to the mobilities of fragments produced by cleavage of primer with S_1 nuclease (Fig. 1, lanes a, b, and c) and concluded that the portion of primer contained in the duplex is 15 to 20 nucleotides in length. This represents approximately 24% of the total tRNA molecule and conforms approximately to the fraction of primer found in RNase-resistant duplexes with viral genome (13 to 19%; Table 2).

Analysis of the nucleotide sequence that binds primer to the ASV genome. A duplex between ^{32}P-labeled primer and unlabeled ASV genome was prepared as described above, using both pancreatic and T_1 RNases to hydrolyze single-stranded RNA. The purified duplex was denatured by heating, hydrolzyed with T_1 RNase, and subjected to two-dimensional electrophoresis on paper according to Sanger et al. (21). The resulting "fingerprint" contained four major oligonucleotides (Fig. 3b); several additional oligonucleotides were present in relatively small amounts (molar yields, <5%), which we attribute to contamination. Three of the oligonucleotides (numbers 1, 2, and 3, Fig. 3d) correspond to oligonucleotides found in the fingerprint of native primer (Fig. 3a); the identity between oligonucleotides 1, 2, and 3 derived from the duplex and their counterparts in native primer was documented further by determination of nucleotide composition and analysis of the products of hydrolysis with pancreatic RNase (Table 3). Using the established nucleotide sequence of primer (Fig. 5), we deduced that oligonucleotide 1 is derived from loop IV of the tRNA, oligonucleotide 2 from the junction between loop IV and the acceptor stem, and oligonucleotide 3 (multiple residues of Gp) from the acceptor stem.

Oligonucleotide 4 had no counterpart in the fingerprint of native primer (Fig. 3a and b). The nucleotide composition and deduced sequence of this oligonucleotide (Table 3) suggested that it was derived from the 3′ terminus of the primer but had lost the adenosine that normally terminates the tRNA. We explored this possibility further by comparing the 3′ ter-

TABLE 3. *Oligonucleotides resulting from RNase T_1 hydrolysis of the portion of primer in a duplex resistant to T_1 and pancreatic RNases (see Fig. 3b)*[a]

Oligonucleotide no.	Molar ratios[b]	T_2 RNase products and relative yield	Pancreatic RNase products and relative molar yields[c]	Deduced sequence[d]
1	1	m^1Ap,Ap,Cp,Up,Gp (1.3,2.1,2.1,0.9,1)	m^1A-A-Up,A-Cp,Cp,Gp (0.9,0.9,1.0,1)	m^1A-A-U-C-A-C-Gp
2	1.2	Up,Cp,Gp (1.0,0.9,1)	Up,Cp,Gp (1.0,0.7,1)	U-C-Gp
3	2.9-3.7	Gp	Gp	Gp
4	1.0	Ap,Up,Cp (1.1,1,3.3)	A-Cp,Up,Cp (1.3,1,3.5)	U-C-A-C-Cp

[a] Heat-denatured [^{32}P]primer-subunit duplex was hydrolyzed with RNase T_1. Electrophoresis in two dimensions was carried out according to the method of Sanger et al. (21). See Fig. 3d for the numbering system. Each oligonucleotide was isolated from the fingerprint and digested with RNase T_2; the resulting mononucleotides were separated by electrophoresis on 3MM paper at pH 3.5. Each oligonucleotide was isolated from the fingerprint and hydrolyzed with pancreatic RNase; the products were separated by electrophoresis on DEAE paper at pH 3.5. Quantitations of the redigestion products were carried out by counting radioactivity in a low-background planchet counter or in a liquid scintillation counter.

[b] Molar ratios of the oligonucleotides were determined relative to oligonucleotide 1, known to be present in only one copy in the tRNA primer.

[c] Gp is taken as 1 in determining the relative molar yields of pancreatic RNase products from the individual oligonucleotides (except oligonucleotide 4, where Up is taken as 1).

[d] Although these data provide only partial sequence analysis, the actual sequence of the oligonucleotides has been determined by complete analysis of the entire tRNA primer (17; our unpublished data).

FIG. 3. *Oligonucleotides produced by hydrolysis with RNase T_1. The isolation of ^{32}P-labeled RNAs, hydrolysis with RNase T_1, and separation of products by two-dimensional paper electrophoresis were carried out as described in Materials and Methods. (a) Purified tRNA primer. (b) Portion of primer found in a duplex with the ASV subunit after hydrolysis with RNase T_1 and pancreatic RNase. (c) Portion of primer found in a duplex with the ASV subunit after hydrolysis with Rnases T_1 and T_2 and pancreatic RNase. (d) Schematic presentation of (b) and (c) and numbering system. The letter B designates the migration of blue dye on each fingerprint.*

mini of oligonucleotide 4 and native primer (Table 4). Oligonucleotide 4 released phosphate when treated with bacterial alkaline phosphatase, whereas primer has an unphosphorylated terminus. Hydrolysis of oligonucleotide 4 with U_2 RNase produced a dinucleotide from the 3′ terminus, which yielded only pC when hydrolyzed with snake venom phosphodiesterase (after prior treatment with bacterial alkaline phosphatase to remove 3′ phosphates). The same procedure released both pC and pA from the 3′ terminus of native primer. We conclude that oligonucleotide 4 terminates in Cp, presumably as a consequence of the nucleolytic removal of the adenosine that terminates native primer.

As a final demonstration of the origin of oligonucleotide 4, we prepared the oligonucleotide U-C-A-C-C-A_{OH} from the 3′ terminus of primer and then subjected it to β-elimination to remove the terminal adenosine. The product was found to be identical to oligonucleotide 4 when analyzed by two-dimensional electrophoresis (Fig. 4) and enzymatic hydrolysis (Table 4).

The amount of cytosine in the oligonucleotide released from the 3′ terminus of primer by hydrolysis with T_1 RNase was too high to conform to the published sequence, U-C-A-C-C-A_{OH} (Table 4); our data suggested the presence of an additional residue of cytosine and the sequence U-C-A-C-C-C-A_{OH}. To resolve this conflict, we treated the oligonucleotide with both U_2 RNase and bacterial alkaline phosphatase to produce either $CpCpA_{OH}$ or $CpCpCpA_{OH}$, depending upon which of the possible nucleotide sequences was correct. The enzymatic product was then chromatographed on DEAE-cellulose according to Tener (25) and found to have a charge of −2 (data not shown); this established the correct sequence as $CpCpA_{OH}$. We suggest that the spurious values for cytosine in our data are due to turnover of the terminal nucleotides in the tRNA in vivo, resulting in a higher specific activity.

Using the established nucleotide sequence for primer (Fig. 5), we can assemble the preceding data into a sequence ($^{m'}$A-A-U-C-A-C-G-U-C-G-G-G-G-U-C-A-C-Cp) present in the 3′ terminal portion of the primer. The duplex containing this sequence was prepared with T_1 and pancreatic RNases, neither of which can hydrolyze RNA at adenosine residues; consequently, the presence of the 5′ terminal $^{m'}$Ap and the penultimate Ap in the sequence is suspect. To better define the 5′ end of the sequence, we prepared duplex with pancreatic and T_1 RNase

TABLE 4. *Nucleotide analysis of 3′ terminal oligonucleotides produced by RNase T_1 hydrolysis*[a]

3′ Terminal oligonucleotide from:	RNase T_2 products and relative yields	Release of $PO_4^=$ by BAP-F	RNase U_2 products and relative yields	Products of complete snake venom phosphodiesterase digestion of RNase U_2* products	Deduced sequence[b]
Native primer, untreated	Cp,Ap,Up (3.9,1,0.8)	−	[U,C]Ap,C-C-AOH* (1.1,1)	pA,pC	U-C-A-C-C-AOH
RNase-resistant duplex (oligonucleotide 4)	Cp,Ap,Up (3.3,1,0.7)	+	[U,C]Ap,C-Cp* (1.1,1)	pC	U-C-A-C-Cp
Native primer, β-eliminated	Cp,Ap,Up (3.7,1,0.7)	ND	[U,C]Ap,C-Cp (1.1,1)	ND	U-C-A-C-Cp

[a] Primer and the segment of primer contained in the duplex were hydrolyzed with RNase T_1, and the products were separated by two-dimensional electrophoresis according to the method of Sanger et al. (21). The 3′ terminal oligonucleotide from the complete tRNA primer (see arrow in Fig. 3a) and the 3′ terminal oligonucleotide from the duplex (no. 4 in Fig. 3c) were isolated. An aliquot of the 3′ terminus originating from the whole primer was subjected to β-elimination with periodate and aniline treatment according to the procedure described by Fraenkel-Conrat and Steinschneider (14). A portion of the treated 3′ terminus was reanalyzed by two-dimensional electrophoresis (Fig. 4), and quantitation of the fingerprint demonstrated the reaction was approximately 90% complete. The treated 3′ terminal oligonucleotide, its untreated counterpart, and the 3′ terminal oligonucleotide from the duplex segment were individually digested with RNase T_2 (and the resulting mononucleotides were separated by electrophoresis on 3MM paper at pH 3.5) and RNase U_2 (and the products were separated by electrophoresis on DEAE paper at pH 1.9). The C-Cp and C-C-AOH products (designated with *) from the duplex segment and native primer, respectively, were further characterized by alkaline phosphatase treatment followed by complete digestion with snake venom phosphodiesterase. The resulting mononucleotides were separated by electrophoresis on 3MM at pH 3.5. Quantitative determinations were made by counting radioactivity in a liquid scintillation counter. ND, Not determined; BAP-F, bacterial alkaline phosphatase.

[b] Although these data provide only partial sequence analysis, the actual sequence of these oligonucleotides has been determined by complete analysis of the entire tRNA primer (17; our unpublished data).

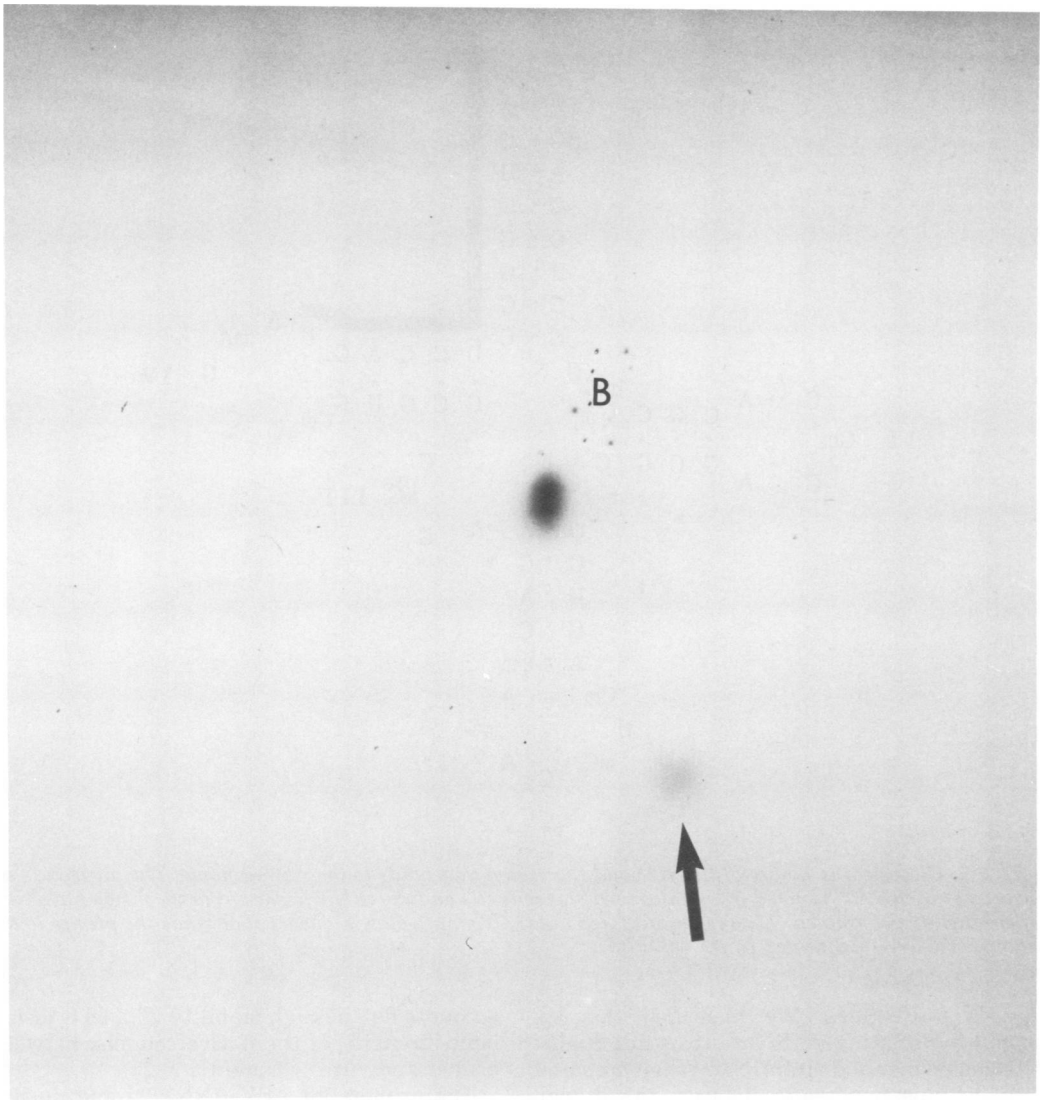


FIG. 4. *Two-dimensional paper electrophoresis of 3' terminal T_1 oligonucleotide of primer after β-elimination. Primer was digested with RNase T_1, and the products were separated by two-dimensional paper electrophoresis. The position of the 3' terminal oligonucleotide is known from previous work (11, 17; see arrow, Fig. 3a). This T_1 oligonucleotide was isolated and subjected to β-elimination according to the procedure described in Materials and Methods. The treated product was reanalyzed by electrophoresis on paper in two dimensions. The arrow marks the position of the untreated 3' terminal T_1 oligonucleotide.*

as before, carried out an additional hydrolysis with T_2 RNase, and then analyzed the nucleotide sequence of the remaining RNA. Hydrolysis of the denatured RNA with T_1 RNase produced four oligonucleotides, as before (Fig. 3c). Three of these were identical to the previously identified oligonucleotides 2, 3, and 4 (Table 5); the fourth proved to be oligonucleotide 1 without the residues of ^{m1}Ap and Ap at the 5' terminus.

We conclude that a sequence of 16 nucleotides binds $tRNA^{trp}$ to the genome of ASV. The location of this sequence in the tRNA molecule is outlined in Fig. 5.

DISCUSSION

The primer for transcription of DNA from the genome of ASV is $tRNA^{trp}$, which is base paired with the viral genome by a sequence of 16 con-

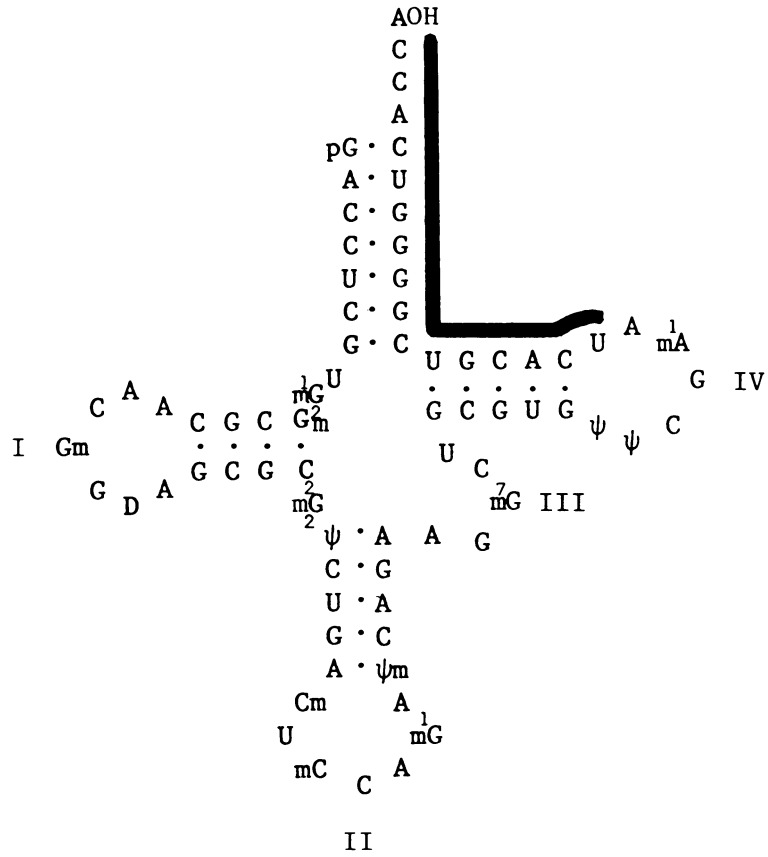


FIG. 5. *Cloverleaf structure of tRNA^{trp} and the region that binds to the ASV genome. The nucleotide sequence of the tRNA^{trp} isolated from uninfected chicken cells and the tRNA primer from the virion have been independently determined (17; our unpublished data). The nucleotide sequence that binds the primer to the genome of ASV is delineated by the solid line.*

tiguous nucleotides. We identified this sequence by isolating an RNase-resistant duplex between primer and viral RNA. When prepared with T_1 plus pancreatic RNases, the duplex extends to the penultimate nucleotide (Cp) at the 3′ end of the primer but does not include the 3′ terminal adenosine. Since the base pair of A:U is not removed from the 5′ end of the duplex by pancreatic RNase or by RNase T_2, we propose that the 3′ terminal adenosine may not be paired with a complementary nucleotide in the viral genome. However, the exclusion of the 3′ terminal adenosine from the duplex may be an artifact of nuclease digestion. If this is not an artifact, the apparent exclusion of the nucleotide from hydrogen-bonded base pairing could be important to the mechanism of initiation since polymerization of DNA initiates on this adenosine.

Approximately 70% of the nucleotides in the binding sequence are either Gp or Cp. This accounts for the high stability (T_m, 69 C at low ionic strength) of the native complex between primer and viral genome (7, 9, 23).

The primers for ASV (tRNA^{trp}) and murine leukemia virus (tRNA^{pro}) contain two adjacent residues of pseudouridine, one of which replaces a residue of ribothymidine usually present in loop IV of tRNA's (11, 17; Dahlberg, personal communication). To date, this unusual pair of modified bases has not been found in any other tRNA and, thus, may represent a functionally important feature of the structure of primer. We have shown that the pseudouridines are not included in the nucleotide sequence that binds tRNA^{trp} to the ASV genome, but it is possible that the modified bases participate in the interaction between primer and RNA-directed DNA polymerase.

The duplex formed between viral RNA and primer includes all but one of the nucleotides present in the acceptor stem of the native tRNA

TABLE 5. *Oligonucleotides resulting from RNase T_1 hydrolysis of the portion of primer in a duplex resistant to T_1, T_2, and pancreatic RNases*[a]

Oligonucleotide no.	Pancreatic RNase products and relative molar yields[b]	Deduced sequence[c]
1[d]	A-Cp,Up,Cp,Gp (0.9,0.8,1.0,1)	U-C-A-C-Gp
2	Up,Cp,Gp (0.9,0.9,1)	U-C-Gp
3	Gp	Gp
4	A-Cp,Up,Cp (1.3,1,3.7)	U-C-A-C-Cp

[a] [^{32}P]primer-subunit duplex produced by digestion with pancreatic RNase and RNases T_1 and T_2 was denatured with heat and digested with RNase T_1. The products were separated by electrophoresis in two dimensions on paper by the method of Sanger et al. (21). Each RNase T_1 oligonucleotide (see Fig. 3c) was further characterized by enzymatic digestion with pancreatic RNase as described in the footnotes to Table 3. The numbering system used here is the same as that in the schematic drawing of Fig. 3d.

[b] See footnote *c*, Table 3.

[c] Although these data provide only partial sequence analysis, the actual sequence of these oligonucleotides has been determined by complete analysis of the entire tRNA primer (17; our unpublished data).

[d] This oligonucleotide migrates to a slightly different position than oligonucleotide 1 in Fig. 3b due to a difference in composition, as demonstrated here.

(Fig. 5). Since the acceptor stem often figures in the specific recognition of tRNA's by synthetase enzymes (8, 15), we hypothesize that the duplex between primer and viral genome serves an analogous purpose by mediating the binding of RNA-directed DNA polymerase and the subsequent initiation of DNA synthesis. The duplex is favorably located for this purpose since it adjoins the 3′ terminus of the primer, the point at which DNA synthesis is initiated. A similar but less extensive duplex (7 rather than 16 base pairs) is present in the acceptor stem of native $tRNA^{trp}$ (Fig. 5) and could account for the high-affinity binding of $tRNA^{trp}$ to RNA-directed DNA polymerase described recently by Panet et al. (20).

The nucleotide sequence that binds primer to the genome of ASV extends through the acceptor stem and into loop IV of the tRNA molecule. Consequently, the base pairing of primer with viral RNA must disrupt the native conformation of the tRNA. We suggest that during maturation of ASV the RNA-directed DNA polymerase binds to primer and sufficiently denatures the tRNA to facilitate its basepairing with the viral genome. This results in the formation of a complete complex of enzyme, primer, and template.

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