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Nucleotide sequence of the restriction fragment Hind F - Eco R_I2 of SV40 DNA

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ABSTRACT

The nucleotide sequence of the SV40 genome region between the Hind K fragment and the Eco R_I cleavage site has been determined by a combination of three different approaches : analysis of RNA products obtained by transcription with Escherichia coli DNA dependent RNA polymerase, partial degradations with snake venom exonuclease and base-specific chemical degradation of 5'-terminal labeled restriction fragments. This nucleotide sequence shows only one open reading frame and allows the deduction of a small segment of the amino acid sequence of VP_1, the major structural protein.

INTRODUCTION

The circular genome of the oncogenic virus SV40 has been divided in map units for which the Eco R_I cleavage site was taken as the zero point (1). Physiologically the genome may be divided in an early region and a late region related to the synthesis of the early T-antigen and the late structural protein VP_1, VP_2 and VP_3 respectively. The initiation site of the VP_1 protein has been localized near the Hind E-K junction (2, 3) and the termination region maps in the Hind G fragment (4,5). Therefore the Hind fragments F and J correspond to internal regions of the VP_1 protein gene. Cleavage of Hind F with the restriction enzyme Eco R_I yields two fragments, a larger Hind F-R 1 (footnote) located to the right and a smaller Hind F-R 2 located to the left of the unique Eco R_I site (1). The nucleotide sequence of the latter fragment is here reported.

Footnote : The abbreviation R refers to the Eco R_I enzyme, when the latter is used as a second enzyme for cleavage of a restriction fragment.

MATERIALS AND METHODS

1. Synthesis of (^{32}P)-labeled RNA

Procedures for preparation of transcripts, either under standard conditions or under conditions of primer-dependence of the oligonucleotide $(Ap)_5A$ have been described previously (6).

2. RNA sequence analysis

The RNA transcript was digested either with T_1-ribonuclease or with pancreatic ribonuclease and mini-fingerprinted as described previously (7). The oligonucleotides were characterised by double digestion (8). Partial hydrolysis with CM-ribonuclease (9) was carried out at 37°C with an enzyme to substrate ratio of 1/40. After 30 minutes, the reaction mixture was chilled and applied directly to a cellulose acetate strip. After electrophoresis at pH 3.5, the material was transferred to a DEAE-cellulose HR 2/15 thin layer plate (Macherey-Nagel & Co, Düren, GFR). In a second dimension, fractionation was obtained by homochromatography using homomix β (7). The partial products were eluted and characterised by T_1-ribonuclease and pancreatic ribonuclease mini-fingerprinting (7, 8).

3. 5'-Terminal labeling of the restriction fragments

Two to five pmoles of DNA fragment were dephosphorylated with bacterial alkaline phosphatase (Worthington BAPF; 50 µg/ml) at 60°C during 30 min in 10 mM Tris-Cl buffer, pH 8.0. The phosphatase activity was destroyed by heating 2 min at 100°C in the presence of 5 mM nitrilotriacetic acid (10) or by phenol extraction. The 5'-terminal labeling was as described previously (3) with T_4-polynucleotide kinase (a gift of Dr. K. Kleppe) and γ-(^{32}P)-ATP (1,000-1,500 Ci/mmol), prepared according to Glynn and Chappel (11) from New England Nuclear carrier-free (^{32}P)-phosphate and P-L. Biochemicals ATP. The work-up of the 5'-terminally labeled restriction fragment was essentially as described earlier (3, 12) although often higher restriction enzyme concentrations were needed to obtain complete digests.

4. DNA sequencing techniques

For partial snake venom exonuclease (Worthington) digestion,

the ethanol-precipitated DNA fragment was dissolved in 4 µl 10 mM $MgCl_2$ and 1µl pancreatic deoxyribonuclease I (DNase I) was added (2 ng/µl in 50 % glycerol, 10 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$). After incubation for 15 min at 37°C the DNase activity was destroyed by heating for 2 min in a boiling water-bath, and 1 µl yeast RNA (30 µg/µl), 3 µl H_2O and 1 µl snake venom phosphodiesterase (1 µg/µl in 50 % glycerol, 300 mM glycine-KOH buffer, pH 9.0) were added. The digestion was at 37°C and 1 µl samples were removed at 15 min time intervals and denatured in 10 µl 1 M NH_4OH + 1 mM EDTA. The pooled samples were dried in an air stream and redissolved in 4 µl standard dye mixture. The fractionation of the digest was by electrophoresis on a cellulose-acetate strip at pH 3.5 in the first dimension and by homochromatography with mixture β (7) at 65°C on Cel 300 DEAE/HR-2/15 thin layer plates (Polygram, Machery-Nagel Co) in the second dimension.

The chemical degradations of the 5'-(^{32}P)-DNA fragments were essentially according to the procedures as communicated to us by A. Maxam and W. Gilbert (13). The results obtained by this procedure were in complete agreement with our nucleotide sequence data derived by transcription into RNA for the Hind H fragment (2, 6), parts of the Hind fragments K(2) and J (H. Van Heuverswyn, personal communication). The fractionation of the products was on 20 % polyacrylamide gels (2 mm x 30 cm x 90 cm) in 8 M urea, 50 mM Tris-acetate pH 8.0 and 2.5 mM EDTA at 1000 V.

5. Other methods

Preparation of SV40 (strain 776) DNA, isolation of restriction fragments and other relevant methodology has been described before (3,6,12).

RESULTS

1. RNA nucleotide sequence data obtained by transcription of the Hind F-R 2 fragment

Figure 1 shows an example of a T_1-RNase and a pancreatic RNase (P) minifingerprint of a Hind F-R 2 primer-dependent transcript. The composition of these fingerprints is very simple,

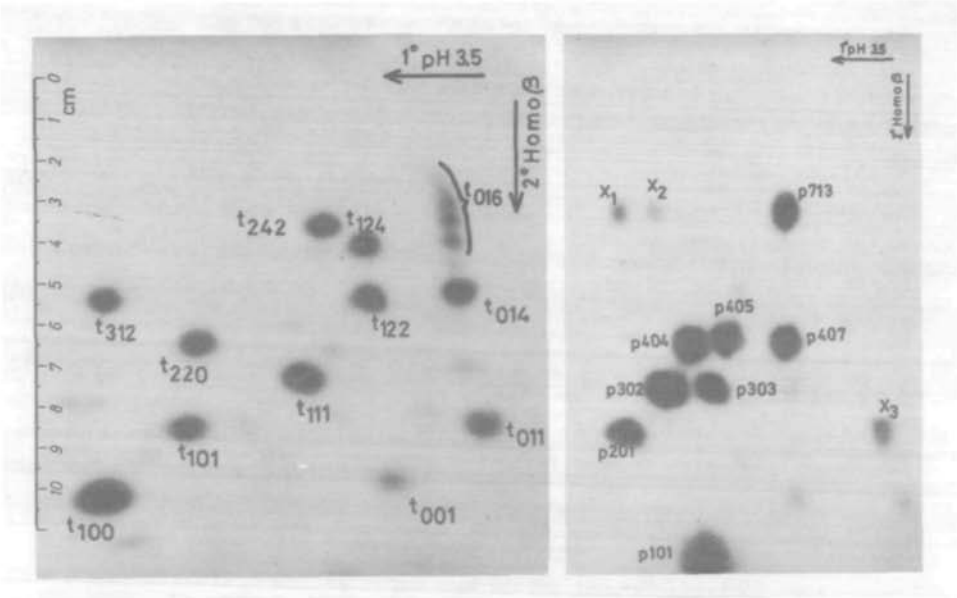

Figure 1 : The $(Ap)_5A$-primer dependent ^{32}transcript was synthesized as described previously (6) using α-(^{32}P)-GTP as the labeled precursor. The product was purified by polyacrylamide gel electrophoresis under denaturing conditions. The main band was cut out, eluted and precipitated with ethanol in the presence of 40 µg carrier RNA. Minifingerprinting was as described before (7).

a. T_1-minifingerprint.
The oligonucleotide numbers refer to their composition (7, 18). (Note that t_{001} is generated by secondary degradation). t_{016} is the 5'-terminal oligonucleotide, present as a series of spots (cf. text).

b. P-minifingerprint.
The numbering is as described before (6), and the analysis is given in Table 2. t_{101} partly ran off of the plate. The origin of some weaker oligonucleotides, indicated by x_1, x_2 and x_3 is not known (upon double digestion, they all contain A-Gp and they do not correspond to oligonucleotides from the Hind F-R 2 minus strand DNA).

in agreement with the small chain length of this DNA-fragment. Under these conditions only one strand is apparently transcribed (except for the aberrant 3'-terminus; vide infra). The T_1-oligonucleotides were characterized by P.RNase digestion and fractionation of the double digestion products on miniplates (8). The results, compiled in Table 1, enable the sequence deduction of all oligonucleotides, except t_{242} in which a C-U sequence can still be interchanged. As will be discussed below, t_{111} has a

Oligonucleotide a* (a)	Molar yield (b)	Double digestion products (c) α-^{32}P-GTP	α-^{32}P-UTP	α-^{32}P-ATP	α-^{32}P-CTP	Sequence deduced
t_{100}	3.1	Up : 1 Gp : 1	Gp	-	-	U-Gp(G) and U-Gp(U)
t_{011}	0.7	A-Gp	A-Gp	Cp	-	C-A-Gp(U)
t_{101}	0.9	A-Up	A-Up	Gp	-	A-U-Gp(A)
t_{111}(d)	1.7	A-Cp	Cp	Up A-Gp (weak)	-	C-U-A-Gp(G) (A)
t_{220}	1.1	Up	Cp : 1 Up : 1	-	Cp : 1 Gp : 1	C-C-U-U-Gp(C)
t_{014}	1.0	A-A-A-Gp	-	A-A-A-Gp : 3 A-Cp : 1	A-Cp	A-C-A-A-A-Gp(A)
t_{122}	1.1	A-Gp	A-Gp : 1 Cp : 1	U : 1 A-Cp : 1	A-Cp	C-U-A-C-A-Gp(U)
t_{312}	1.0	A-Gp	Up	A-Gp : 1 A-Cp : 1 Up : 1	A-Cp	U-U-U-A-C-A-Gp(A)
t_{124}	0.9	Up	A-A-Cp	A-A-Cp	A-A-Cp Gp	A-A-C-A-A-C-U-Gp(C)
t_{242}	1.0	A-Cp	A-Cp : 1 Cp : 1	A-Cp : 1 Cp : 1	A-Cp : 1 Cp : 1 Up : 2	A-C-U-C(C,U)C-A-Gp(A)
t_{016}(e)	N.D.	A-Gp	A-Gp	+	+	A_{5-8}C-A-Gp(U)

TABLE 1 : RNase T_1-oligonucleotides.

(a) The oligonucleotide numbering corresponds to a standardized system (7, 18) based on the nucleotide composition.

(b) The yield is expressed as molar equivalents of radioactivity upon α-(^{32}P)-GTP labeling.

(c) Whenever more than one double-digestion product was present, the relative amounts were estimated by visual inspection of the double digestion miniplates (8).

(d) The presence of A-Gp in the double digest of the α-(^{32}P)-ATP labeled oligonucleotide and the radioactive equivalence of 1.7 in the α-(^{32}P)-GTP labeling experiment suggest an heterogeneity in the nearest neighbour of this oligonucleotide. Clear-cut evidence was obtained from the analysis of a CM-RNase partial digest of the RNA-transcript.

(e) t_{016} is the 5'-terminal oligonucleotide of the transcript, present as a series of spots with increasing number of Ap residues (6). The oligonucleotide was not further analysed in the α-(^{32}P)-ATP and the α-(^{32}P)-CTP experiments.

N.D. = not determined.

second nearest neighbour present to a lesser extent. The sequence of the T_1-oligonucleotides which contain one or more A-residues has been confirmed by U_2-RNase digestion (data not shown).

P-oligonucleotides labeled by α-(^{32}P)-GTP were characterized with T_1-RNase. As the base composition of these oligonucleotides was known from their position on the fingerprint, the primary structures could be derived from this single GTP-labeling experiment. Fingerprints of the ATP-, UTP- or CTP-labeled products, in conjunction with the T_1-oligonucleotide sequence data, allowed the deduction of all the nearest neighbours as shown in Table 2.

Oligonucleotide n° (a)	Molar yield (b)	Double digestion products (c) α-^{32}P-GTP	α-^{32}P-UTP	α-^{32}P-ATP	α-^{32}P-CTP	Sequence deduced (d)
P_{101}	N.D.*	Up	+	+	+	Up
P_{102}	-	-	+	+	+	Cp
P_{201}	1.0	Up	+	-	-	G-Up(G)
P_{203}	-	-	+	-	+	G-Cp(U)
P_{204}	-	-	-	+	+	A-Cp(A)
P_{302}	3.7	A-Gp : 2(3)(e)	+	-		A-G-Up(G) + A-G-Up(U)
		Up : 1				
P_{303}	1.0	Gp	+	-	+	G-G-Cp(U)
P_{305}	-	-	+	+	+	G-A-Cp(U)
P_{306}	-	-	+	+	+	A-A-Cp(U)
P_{404}	1.9	A-Gp : 1 A-Up : 1	+	+	-	A-G-A-Up(G)
P_{405}	1.1	A-Gp	-	+	+	A-G-G-Cp(A)
P_{407}	1.2	A-Gp	-	+	+	A-G-A-Cp(A)
P_{713}	1.0	A-A-A-Gp	-	+	+	A-A-A-G-A-A-Cp(A)

TABLE 2 : P-RNase oligonucleotides.
(a) The numbering of the oligonucleotides is as described previously (6).
(b) Based on an experiment with α-(^{32}P)-GTP as labeled precursor.
(c) Double digestion was performed in the α-(^{32}P)-GTP experiment only. In the other experiments, the presence was deduced by visual inspection of a P-RNase minifingerprint (8).
(d) Nucleotide sequence and nearest neighbour was deduced in combination with the results obtained on the T_1 RNase oligonucleotides.
(e) Estimation by visual inspection.
N.D. = not determined.

Additional confirmation for both oligonucleotide catalogues was provided by some experiments in which the transcription had been carried out under "standard" conditions (6) (data not shown).

Ordering of oligonucleotides

The 5'-terminal oligonucleotide of an $(Ap)_5A$-primer-dependent transcript is easily detected by its typical, multiple position on the mini-fingerprint (a varying number of Ap-residues at the 5'-terminus) (6). In the present situation, this initiating tract is found in the first graticule (oligonucleotides containing no Up-residues). With the exception of C-U-A-Gp and C-A-Gp (vide infra), all T_1-oligonucleotides are present in one molar yield. Some overlaps can be deduced by comparison with the P-oligonucleotides, e.g. A-C-A-A-A-Gp(A) is linked to A-A-C-A-A-C-U-Gp(C) by the overlap A-A-A-G-A-A-Cp(A).

Partial digestion of the transcript with CM-RNase has been used successfully to order the remaining oligonucleotides. Under the conditions described in the section Materials and Methods, a set of partial products was isolated and characterised. The results are illustrated in figure 2, which also shows the entire nucleotide sequence of the RNA transcript. The chain length and the oligonucleotide composition of the partial products are in agreement with their position on the map.

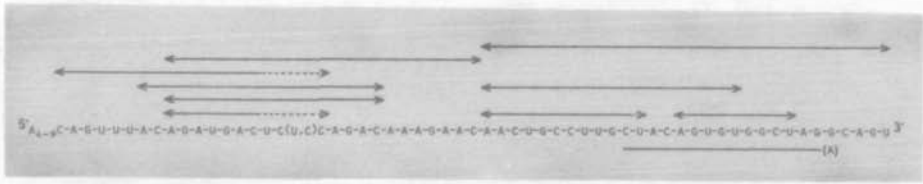

Figure 2 : Nucleotide sequence of the $(Ap)_5A$ primer-dependent transcription product of the Hind F-R 2 fragment.
Line segments above the sequence indicate the partial products obtained by limited digestion of the transcript with CM-RNase as described in the text. Broken lines are regions not labeled in that particular experiment but expected to be part of the partial product on the basis of the specificity of CM-RNase (9) and in agreement with the position of the fragment on the two-dimensional fractionation pattern.
The A-residue between brackets (below the sequence) refers to the heterogeneity discussed in the text.
The underlined sequence was confirmed by wandering spot analysis of the corresponding, complementary DNA-segment (Fig. 3a).

The T_1-oligonucleotides C-U-A-Gp and C-A-Gp, joined by the overlapping P-oligonucleotide A-G-G-Cp, are located at the 3'-terminal region. The molar yield of these T_1-oligonucleotides amounts to 80 % and 65 % respectively, suggesting that not all termination occurs at a single position. It is also obvious that the transcription does not proceed to the physical end of the DNA-fragment, as no oligonucleotides corresponding to the Hind III or Eco R_I recognition sites are present.

Moreover, double digest analysis of the t_{III}-oligonucleotide obtained from an α-(^{32}P)-ATP labeled transcription reaction, revealed an heterogeneity in the nearest neighbour of this oligonucleotide. Indeed, to some extent an Ap is detected instead of Gp. The meaning and consequences of this pecularity became clear after sequence analysis at the DNA level as discussed below.

2. Nucleotide sequences at the 5'-ends of the Hind F-R 2 fragment obtained by two-dimensional fingerprinting of partial venom exonuclease digests

The preparation of the Hind F-R 2 fragments only 5'-labeled in one of both strands was obtained as follows. The restriction fragment Hae B-R 2 (i.e. the smaller of two fragments, obtained by treatment of Hae B with Eco R_I; cf. ref. 12) was terminally labeled and then digested with the Hind II + III enzyme mixture; this gave us the fragment Hind F-R 2, labeled in the plus strand only. Kination of the Hind F fragment, followed by digestion with the Eco R_I enzyme, resulted in the Hind F-R 2 fragment labeled in the minus strand only. The partial snake venom exonuclease digest of the fragment with the (^{32}P)-label at the Eco R_I-cleavage site is shown in figure 3a. The wandering spot pattern allows the deduction of the sequence
5'... p(Np)n TpCpTpApGpCpCpApCpApCpTpGpTpApGp... . This sequence is complementary to the 3'-terminal region of the RNA segment represented in figure 2 (indicated by underlining) except for the last 5 nucleotides. Furthermore, it contains 7 identical nucleotides out of an 8 nucleotide stretch of the sequence spanning the Eco R_I site of SV40 as determined by Garfin et al. (14). The mobility shift observed in our system for the non-identical nucleotide strongly suggests an Ap-residue (19) rather than a Cp-residue. The identification of this only different nu-

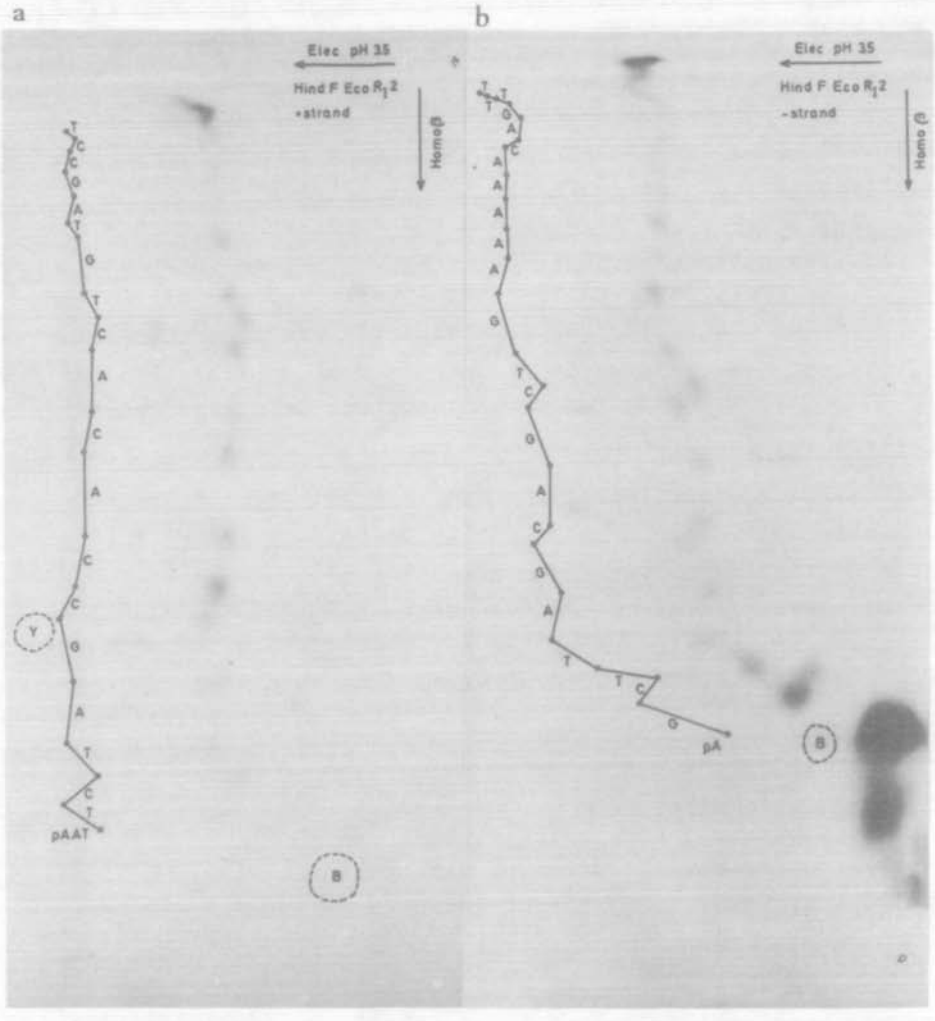

Figure 3 : Two-dimensional fingerprints of partial exonuclease digests of 5'-labeled fragments.
The 5'-terminally labeled fragment was treated in 5 µl 5 mM Tris-Cl + 0.5 mM $MgCl_2$, pH 7.4, with 2 ng pancreatic DNase for 15 min at 37°C. 1 µl containing 30 µg yeast-RNA, 3 µl H_2O and 1 µl venom exonuclease in 0.3 M glycine-KOH buffer, pH 9.0, were then added. One µl samples were taken at 15 min time interval; the pooled samples were then subjected to two-dimensional fingerprinting.

cleotide as an Ap-residue is further established by the chemical degradation pattern of this region (see figure 4) and by the RNA nucleotide sequence data shown in figure 2.

The two-dimensional fractionation of the partial exonuclease digestion of the Hind F-R 2 fragment, (^{32}P)-labeled at the Hind III site (minus strand) allows the deduction of the sequence 5'... pApGpCpTpTpApGpCpApGpCpTpGpApApApApApCpApGpTpTpTp (Fig. 3b). This region connects the 5'-end of the fragment with the 5'-end of the $(Ap)_5$A-primed transcript shown in figure 2 and forms an overlap of more than 6 nucleotides. The chemical degradation pattern of this DNA strand, although technically slightly imperfect in the T + C lane (data not shown), fully supports the nucleotide sequence deduced from the wandering spot. Fig. 3b illustrates clearly the presence of an $(Ap)_5$ tract, from where, in the $(Ap)_5$A-primer-dependent transcription, the RNA chain is initiated.

3. Chemical degradation of the Hind F-R 2 (plus strand)

Figure 4 shows the degradation pattern of the Hind F-R 2 fragment labeled at the Eco R_I-cleavage site. The lane G shows the cleavage products at dG-residues, fractionated according to chain length, obtained from the methylated DNA after depurination and hydrolysis with 1M piperidine at 90°C (13). The lane A + G shows the cleavage products of the same methylated DNA after depurination in 20 mM sodium phosphate, pH 7.0, 5 mM EDTA and chain cleavage in 0.1 N NaOH at 90°C. The treatment of the DNA with hydrazine results in elimination of dT- and dC-residues in a salt-free solution or only in dC-elimination when the reaction is carried out in the presence of 1M NaCl. The nucleotide sequence can be read directly from the autoradiogram, shown in figure 4, from the fourth nucleotide from the 5'-end to the end of the $(Ap)_5$ stretch. Together with the information described in the previous section, the complete nucleotide sequence of the fragment Hind F-R 2 is thus established. The results in figure 4 show also an overlap of 7 nucleotides with the Garfin et al. (14) sequence around the Eco R_I-site in SV40 DNA, and confirm the RNA sequence of figure 2 except for the five 3'-terminal nucleotides (cf. Discussion). It also solves the uncertainty in the sequence of the ribonuclease T_1-oligonucleotide A-C-U-C-U-C-C-A-Gp (Fig. 2).

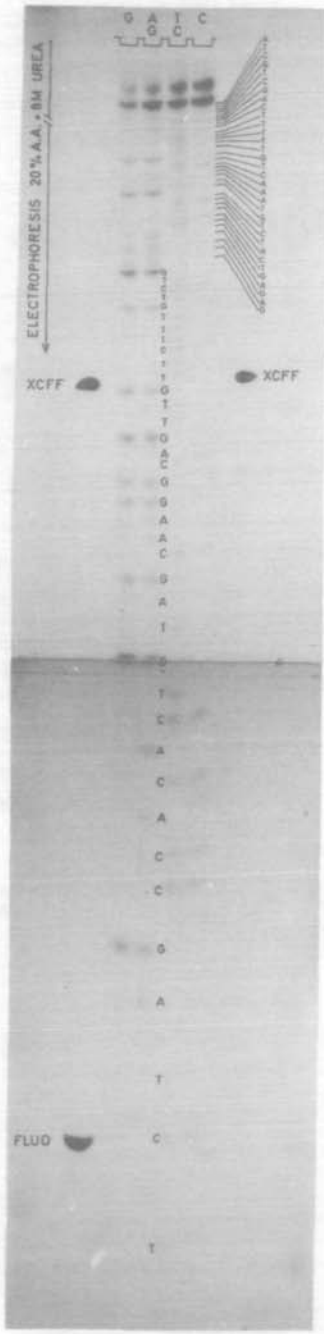

Figure 4: Chemical degradation pattern of the Hind F-R 2 fragment (plus strand).
The 5'-(^{32}P)-labeled (plus strand) Hind F-R 2 fragment was subjected to four base-specific degradation procedures as developed by Maxam and Gilbert (13). Each of the four reaction mixtures was fractionated on a 20% acrylamide gel containing 8 M urea + 50 mM Tris-citrate + 2.5 mM EDTA, pH 8.0, at 1,000 V. The gel was autoradiographed during several days at -20°C. The smallest four 5'-terminal degradation products were run off the gel in this analysis. The designation of the eight upper bands relies on the nucleotide sequence obtained by the wandering spot method (Fig. 3b) and the chemical degradation pattern of the opposite strand (data not shown).

DISCUSSION

We have previously identified the initiation codon of the VP_1-gene in Hind K (3) and, considering the size of the VP_1-protein which is about 49,000 dalton (4, 15), it is clear that the fragment Hind F-R 2 constitutes an internal segment of the VP_1-gene. The nucleotide sequence of the fragment as illustrated in figure 5 contains four termination signals for protein synthesis in the strand corresponding to the sequence of the late mRNA (transcribed clockwise from the plus strand). Three are in the same reading frame but the fourth is in a different reading frame leaving only one frame free for translation into protein. Therefore the amino acid sequence shown in figure 5 must correspond to a segment of the VP_1-protein.

The nucleotide sequence contains the recognition site for the Arthrobacter luteus endonuclease Alu I, only four base pairs away from the Hind III-cleavage site. This is in good agreement with the restriction map determined by Yang et al. (16). Noteworthy is the resistance of this Alu site against cleavage by double digestion of Hind F with Alu enzyme. Indeed, in conditions where the two other Alu sites in Hind F are cleaved completely, almost no cutting at this site is observed. Presumably this is due to its location close to the physical end of the

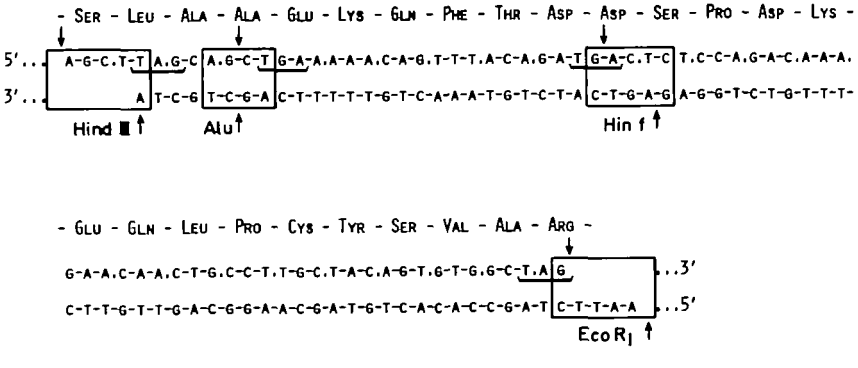

Figure 5 : Nucleotide sequence of the Hind F-R 2 fragment with the amino acid sequence corresponding to an internal segment of the VP_1-protein. The out-of-phase termination codons and the recognition sites of the restriction enzymes Hind III, Eco R_I, Alu and Hin f are also indicated.

Hind F or Hind F-R 2 restriction fragment, but other explanations are not excluded. Recently, Greene et al. (17) have demonstrated that Eco R_I restriction enzyme acts on an octamer, i.e. a double stranded fragment containing only one additional base pair on each site of the recognition site. Therefore at least the Eco R_I enzyme does not seem to be hindered by a physical end in the close vicinity of its recognition site. The Hinf recognition site in Hind F-R 2 is G-A-(C/G)-T-C.

As in our earlier experiments (6) we find that primer-dependent transcription is predominantly asymmetric, giving a nearly homogeneous copy of one strand only. However, the $(Tp)_5$-stretch, to which the $(Ap)_6$A-primer associates for initiation of transcription, is 9 nucleotides away from the 3' end of the (minus) strand. Therefore this terminal region of the fragment is not transcribed under these conditions. The 5'-end of the transcript consists of a homologous series of $(Ap)_4$- to $(Ap)_9$-containing sequences, all derived from the same T_1-oligonucleotide. The shorter ones may either be due to some degradation of the $(Ap)_5$A-primer or to primer-independent transcription, while the longer ones are presumably generated by slippage of the primer or the polymerase during initiation. The five 3'-nucleotides of the transcript do not correspond in a straightforward way to the sequence of the DNA template. In fact, the RNA is transcribed until the end of the double strand while the protruding T-T-A-A remains mainly untranscribed. However, transcription continues and the sequence G-C-A-G-U is further added to the transcript, presumably by copying this segment from the opposite strand. This situation is very similar to what we have observed before with primer-dependent transcription of restriction fragments (6). A possible explanation is that the E.coli polymerase, when it reaches the end of a fragment, still lacks a proper termination signal, and can only release the product (and perhaps the template) by synthesizing a short termination loop; this would involve additional copying of about 5 nucleotides from the displaced, opposite strand. Some RNA-polymerase molecules, however, presumably do not turn to the other strand at this position, and this may explain the heterogeneity in nearest neighbour (85 % CUAG(G) and 15 % CUAG(A); cf. Fig. 2).

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