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Characterization of the genomic RNA from a Rous sarcoma virus mutant temperature sensitive for cell transformation

Jean-Luc Darlix, Mireille Levray, Peter A.Bromley and Pierre-François Spahr

Département de Biologie Moléculaire, Université de Genève, 30, quai Ernest-Ansermet, CH-1211, Genève 4, Switzerland

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ABSTRACT

We have found that the LA23 t/s mutant of Rous sarcoma virus (phenotype Prague B), even when passaged repeatedly at high multiplicity of infection, does not give rise to transformation defective deletion mutants comparable to those derived from RSV. In view of this fact and of the high rate of production of this mutant at 41°C, we have undertaken a detailed analysis of the genome of this virus by ordering all large T_1 oligonucleotides and by determining their nucleotide sequences. The results indicate a high degree of mutation in the *onc* gene as compared to that of Pr-A or Pr-B.

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INTRODUCTION

Rous sarcoma virus mutants that are temperature sensitive for cellular transformation *in vitro* (1-3) are presently being used to study the process of cell transformation by RNA tumour viruses. In our studies we have employed the subgroup B variant of the LA23 mutant isolated by J. Wyke (1) for the infection of chick cells at 41°C and 35°C, the non-permissive and permissive temperatures for transformation by this mutant. We have found that the yield of virus grown at 41°, as estimated by 70S RNA content, is three to four fold higher than that obtained using wild-type Prague B RSV. Furthermore an examination of fingerprints of LA23 70S RNA obtained from virus produced after multiple passages at either low or high multiplicity at 41° and at 35°C showed no indication of the presence of transformation-defective (td) deletion mutants of LA23. This result is in sharp contrast to the published observations indicating that wild-type RSV rapidly yields a mixed progeny of wild-type and td virus after high-multiplicity passaging of the

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virus (4).

In view of this surprising observation we have performed a detailed characterization of the genome of LA23, including the physical mapping of 38 large T_1-oligonucleotides derived from 70S RNA and the elucidation of their nucleotide sequences.

MATERIALS AND METHODS

Cells and viruses. The Prague B and a temperature sensitive mutant for cell transformation [LA23] subgroup B obtained from J. Wyke (Imperial Cancer Research Foundation, London) were grown as reported previously (5).

To look for transformation defective (td) particles of LA23, cell cultures were prepared on petri dishes and infected at low (about 0.1 PFU/cell) and at high (about 10 PFU/cell) multiplicities. Medium was changed every day and collected after 3 days of culture in order to reinfect cells. After three and twelve such passages medium was collected twice and the virus purified by differential centrifugation.

Isolation of virion nucleic acids. RSV 70S RNA and (^{32}P) or (^{3}H) labelled RSV 70S RNA were prepared as already described (5).

Fingerprint analysis. Analysis of the products of T_1 digestion of RSV RNA was performed using two-dimensional polyacrylamide gel electrophoresis (4, 6). The digestion products, uniformly labelled with (^{32}P) or 5' end labelled with ATP (γ-^{32}P) and T4 polynucleotide kinase (7), were located by direct autoradiography, and the radioactivity in large T_1 oligonucleotides was quantitated as previously described (4, 8).

Sequence analysis of the large T_1 oligonucleotides. Unlabelled LA23 RNA (5 ug) was extensively digested with T_1 RNase (Sankyo) and the large T_1 oligonucleotides recovered by precipitation with 2 volumes of ethanol. They were put into 30 µl of a solution containing 50 mM Tris-HCl pH8, 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol and 1.5 mM spermine. After 5 min at 37°C, the 30 µl reaction mixture was transferred to a tube containing dried (γ^{32}P) ATP (25 ci/mole, 2 nanomoles), 1 unit of polynucleotide kinase was added and the reaction continued for a further 10 min.

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Reaction was stopped with 10 mM EDTA and 0.1% sodium dodecyl sulfate. The large T_1 oligonucleotides were purified by two dimensional polyacrylamide gel electrophoresis (6).

To sequence the T_1 oligonucleotides we followed the method described by Donis-Keller et al. (9) to map adenines and pyrimidines in the RNA, and we used both the "wandering spot" analysis (10), Physarum I (11), and pancreatic RNases (12) to distinguish between uridines and cytidines. The 5' end was determined by paper electrophoresis of a total alkali digest of the 5' (^{32}P) end labelled large T_1 oligonucleotides.

RESULTS

Characterization of LA23 RNA. The two-dimensional gel fingerprint pattern of the total T_1 RNase digest of uniformly labelled (^{32}P) LA23 RNA is shown in figure 1a. The fingerprint of LA23 RNA, where the T_1 oligonucleotides were labelled at their 5' end with (^{32}P) ATP and T_4 polynucleotide kinase, is almost identical (fig. 1b) except that T_1 oligonucleotides 10a, 10b, 13a and 13b are better resolved.

Each large T_1 oligonucleotide was sequenced as described in Materials and Methods. An autoradiograph of a sequencing gel of the T_1 oligonucleotides 14a, 14b, 14c and 15 is presented in Fig. 2. Figure 3 reports the "wandering" spot analysis of T_1 oligonucleotides 3, 4, 9 and 20.

Table 1 lists the sequence of all large T_1 oligonucleotides amounting to a total of 635 nucleotides.

Mapping of the T_1 oligonucleotides of LA23 RNA. The mapping was done with both uniformly labelled (^{32}P) LA23 RNA and 5' end-labelled T_1 oligonucleotides of LA23 RNA and gave identical results. The mapping using 5' end-labelled RNA is described below.

After heat denaturation (3 min at 100°C) [LA23] RNA (50 µg) was chromatographed twice on an oligodT cellulose column, and 30% of the input material was recovered as poly(A)$^+$ RNA. The poly(A)$^+$ RNA was then centrifuged through a formamide sucrose

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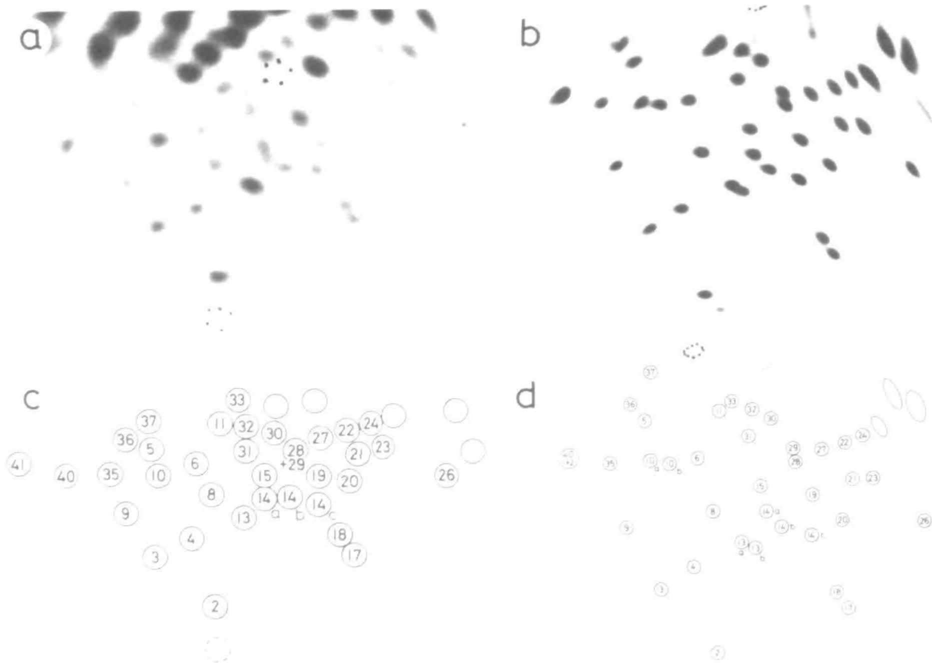

Figure 1

Fingerprint analysis of in vivo and in vitro labelled (^{32}P) LA23 RNA

(a) Uniformly labelled (^{32}P) LA23 RNA was completely digested with T_1 RNase and fingerprinted as mentioned in Methods. Autoradiography was for 24 h. (b) LA23 RNA (5 µg) was digested with T_1 RNase and the T_1 oligonucleotides were labelled at their 5' end with (γ^{32}P) ATP and T_4 polynucleotide kinase as described under Methods. Fingerprint was as in (a) except that electrophoreses were run in gels twice larger. Autoradiography was for 10 min. The T_1 oligonucleotides labelled at their 5' end with ^{32}P were eluted, recovered and their sequence determined (see fig. 2 and 3). (c) and (d) are the schematic representation of respectively (a) and (b).

gradient (Figure 4) and 1 µg of 3' terminal fragments sedimenting at 12-16S, 18-22S, 24-28S, 26-28S, 28-30S and 30-32S were digested with T_1 RNase and the large T_1 oligonucleotides labelled at their 5' end with (γ^{32}P) ATP and T4 polynucleotide kinase and analyzed on two-dimensional polyacrylamide gels.

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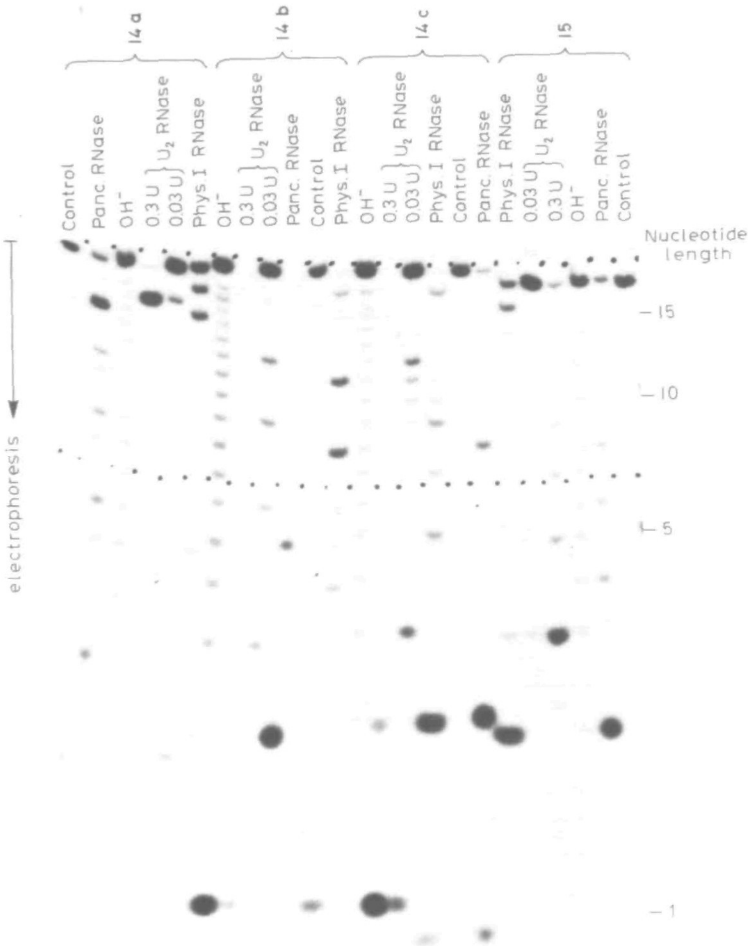

Figure 2

Sequence of T_1 oligonucleotides 14a, 14b, 14c and 15

The sequencing gel (25 % acrylamide, 0.83 % bisacrylamide) was prepared, preelectrophoresed and run as described by Maxam and Gilbert (18). For each reaction 10,000 cpm of (^{32}P) oligonucleotide were used. Autoradiography was for 2 days at -20°C using an NS5T Kodak film. The position of A residues is known from partial U_2 RNase digestions (0.3 and 0.03 unit) and limited alkaline hydrolysis of RNA (90°C, 30 min, pH9) as described by Donis-Keller et al. (9). Partial pancreatic RNase (0.2 ng) and physarum I RNase (0.034 unit, ref. 11) digestions give the positions of C and U residues (12).

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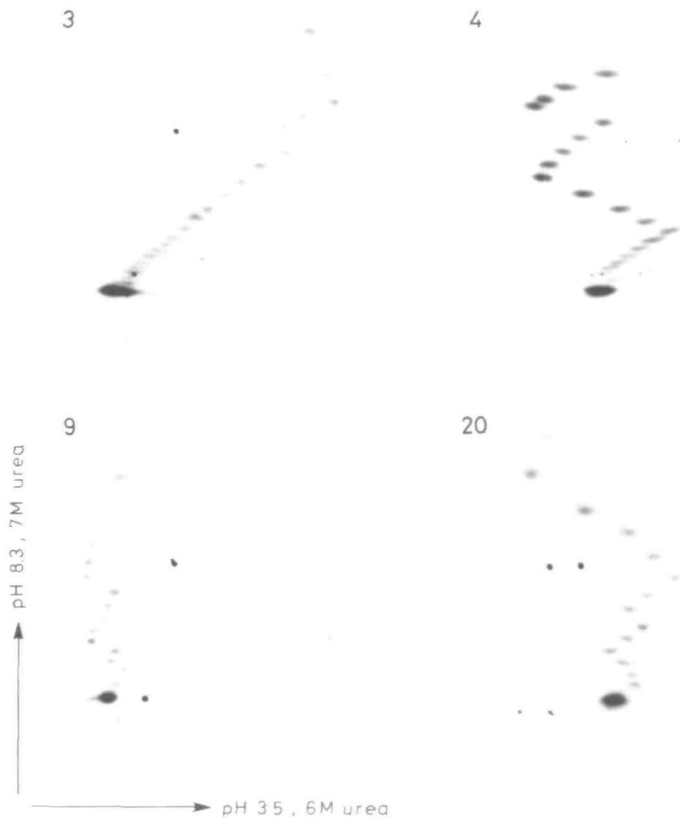

Figure 3

Wandering spot analysis of T_1 oligonucleotides 3, 4, 9 and 20

Positions of C and U residues of T_1 oligonucleotides 3, 4, 9 and 20 known from partial digestions with physarum I and pancreatic RNases (19, 12) were confirmed by wandering spot analyses. Limited alkaline hydrolysis of 10,000 cpm of (^{32}P) oligonucleotide was performed as described in legend to figure 2. The partially hydrolysed oligonucleotides were analyzed by 2 dimensional polyacrylamide gel electrophoresis. First dimension was an electrophoresis on a 10 % polyacrylamide gel at pH 3.5, the second on a 25 % polyacrylamide gel in trisborate-EDTA pH 8.3. Autoradiography was for 3 days at -20°C using an NS5T Kodak film. The positions of A residues is known from U_2 RNase digestions performed and analyzed as described in legend of Fig. 2. Positions of U and C residues are deduced from their relative migration (10). Shift to the right or to the left corresponds to A and C residues or C and U residues, respectively.

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Table 1 Sequence of the large T_1 oligonucleotides from Pr-B [LA23] RNA

Number	Length (nucleotides)	Sequence
2	29	$UCUCUC_4A_2UAUA_2CUA_2UAU_2ACUCG$
3	24	$U_2AU_2CUC_2AC_3A_2C_3AC_2A_2G$
4	22	$C_2AC_2UC_4U_4CA_3CAUG$
5	15	$A_5CA_2U_2ACUAG$
6	16	$AC_2AU_2CAC_2ACAU_2G$
8	18	$A_4C_2UCUACU_2CUA_2G$
9	19	$CACA_2C_3UCAC_2UAUCAG$
10a	16	$AC_2ACAUCACUC_2UCG$
10b	17	$UCC_2AC_4AC_2A_3UG$
11	14	$C_2UCUA_2CUAUACG$
13a	20	$UAUC_3AUC_4UAU_3C_2G$
13b	20	$U_2CA_2UCA_2CUAUCAUACUG$
14a	18	$CUC_2UC_2UC_2UC_2U_2AUG$
14b	18	$ACAUCACUAU_2AU_3CAG$
14c	18	$AUAU_2CUCA_4U_2CU_2G$
15	17	$U_2ACACACAUC_2U_2CUG$
17	21	$U_2A_2U_2AUAU_2CUCAU_2AU_2G$
18	20	$CU_2AU_4CUAUC_3UCU_2G$
19	16	$A_2CA_2UC_2U_3CU_3G$
20	16	$CA_2U_4C_2UCAU_3G$
21	15	$C_2U_4ACA_2U_3CG$
22	14	$UA_2UAUAUCUAUCG$
23	14	$C_2AUCUAU_5CG$
24	13	$U_3ACUAU_3C_2G$
26	12	CU_4AU_4AG
27	15	$UCAUA_2UCAUC_2UCG$
28	15	$CACA_2UCUCU_3AUG$
29	15	$U_2CUC_3ACUAUCUG$
30	14	$C_2U_2AUAU_2C_4G$
31	15	$C_3UCA_2UCACU_3G$
32	14	$U_2A_2UCA_2UCA_2CG$
35	16	$CUA_2CAUAC_3UAC_2G$
36	14	$UC_2ACAUAC_3A_2G$
37	13	UAC_7ACUG
38	13	A_3UC_8G
40a	15	$CAC_4AC_2AUACAG$
40b	15	$C_2ACAC_2UA_4C_2G$
41	12	CAC_6AC_2C

Total 665 nucleotides. One should remember that the nucleotide preceding the 5' terminal nucleotide of each large T_1 oligonucleotide is G. Sequence of the large T_1 oligonucleotides has been determined as described in figures 2 and 3 except for no. 41 where the "wandering spot" experiment was not done.

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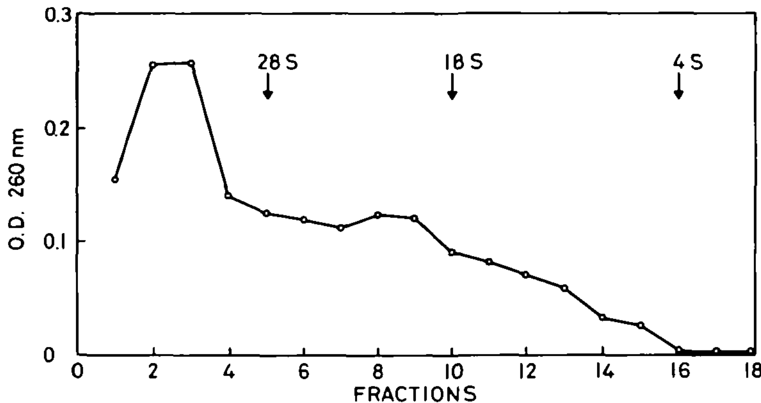

Figure 4

Size fractionation of LA23 poly(A)$^+$ RNA

50 µg of poly(A)$^+$ RNA were layered on a 5 to 20 % sucrose gradient (International, SB283 rotor) in 50 % formamide, 0.1 M LiCl, 5×10^{-3} M EDTA. 0-2 % SDS and 10^{-2} M Tris-HCl pH 7.4. Centrifugation was at 37,000 rpm, for 19 h at 4°C. A gradient containing total cytoplasmic RNA as markers was run in parallel. 18 fractions were collected and O.D. at 260 nm was determined.

Figure 5 shows the fingerprint of the different size class poly (A)$^+$ RNA. The spots corresponding to the large T_1 oligonucleotides were excised from each fingerprint and their radioactivity measured. If the large T_1 oligonucleotides of LA23 RNA are equally abundant and labelled at their 5' end with the same efficiency, the radioactivity should be the same for all the large T_1 oligonucleotides; this was observed except for oligonucleotides 17, 26 and 41 which were consistently found less radioactive (30-40%).

The position of each large T_1 oligonucleotide on the RNA relative to the poly(A) has been first estimated by ranking groups of oligonucleotides after visual inspection of the individual fingerprints (Figure 5 and table 2). Measurement of the radioactivity associated with each oligonucleotide in each size class allowed us to estimate more accurately the position of each large T_1 oligonucleotide on LA23 RNA relative to the poly(A) (fig. 5 and table 2). Finally the oligonucleotide 6 has been independently mapped within the last 5' end 100 nucleotides (13).

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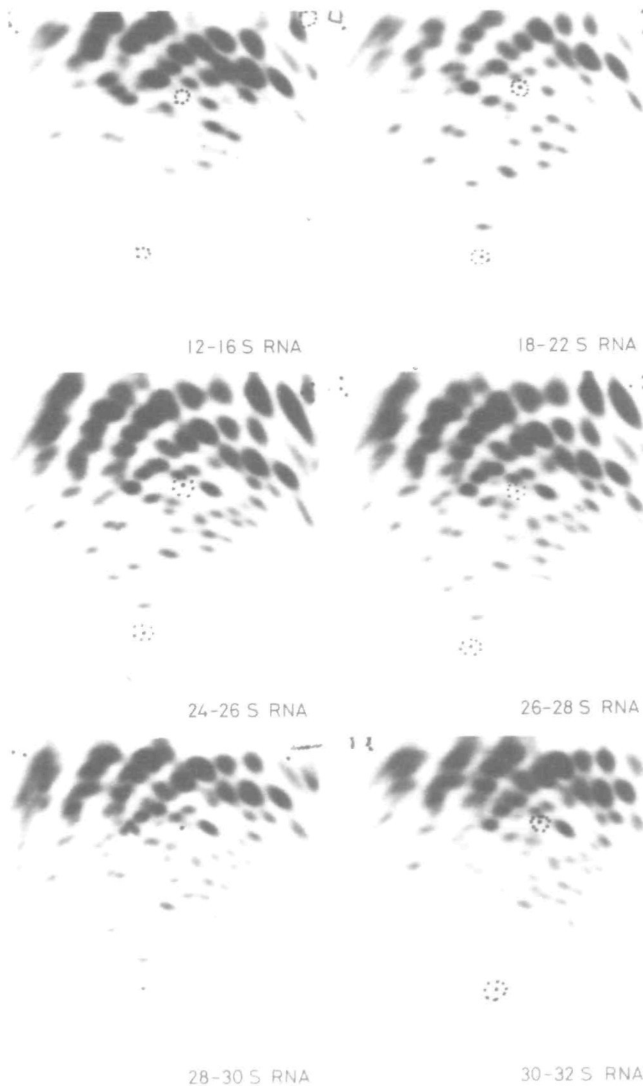

Figure 5

Fingerprints of poly(A)$^+$ containing fragments of LA23 RNA

1 µg of the various size class Poly(A)$^+$ RNA corresponding to fractions 3 (30-32S) (a), 4 (28-30S) (b), 5 (26-28S) (c), 6 (24-26S) (d), 8 and 9 (18-22S) (e) and 11 and 12 (12-16S) (f) was extensively digested with T_1 RNase, the oligonucleotides labelled at their 5' end with (^{32}P) ATP and T_4 polynucleotide kinase (see Materials and Methods) and fingerprinted. Autoradiography was for 2 hours using Kodak NS5T film.

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Table 2 Order of the large T_1 oligonucleotides on LA23 RNA

By visual estimation+	5'end. 6, (30, 35), (8, 14a)/(14b, 40b), (5, 29)/17, (26, 24)/(3, 22, 23, 36), (18, 41)/(31, 37, 38), 13a, (2, 4, 9, 15)/(11, 28, 19, 20), (10b, 13b, 32), (40a, 10a, 14c), 21, 27, poly(A)
After quantitation of the radioactivity in each T_1 oligonucleotide++	5'end. 6, 30, 35, 8, 40b, 14a, 14b, 29, 5, 17, 26, 3, 36, 23, 24, 22, 41, 38, 18, 31, 37, 13a, 4, 9, 15, 2, 19, 11, 28, 20, 10b, 13b, 32, 40a, 10a, 14c, 21, 27, poly(A)

+Inferred by examination of the fingerprints presented in fig. 5 Results are identical with uniformly labelled (^{32}P)LA23 RNA.
Bars indicate a switch from the fingerprint of one subgenomic poly(A) LA23 RNA to the next one, of lower molecular weight, if we start at the 5'end with that of 30-32S RNA. The order of oligonucleotides within parentheses is undetermined.
++Inferred by ranking the relative yields of the large T_1 oligonucleotides for each size class of subgenomic poly(A)LA23 RNA. For example, the radioactivity associated with the large T_1 oligonucleotides of 12-16S poly(A) RNA was in the following increasing order : 3 00 cpm (11, 19 & 20), 600 cpm (28), 2 800 cpm (10b), 3 300 cpm (13b), 3 600 cpm (32), 4 000 cpm (40a), 4 200 cpm (10a), 4 700 cpm (14c), 8 500 cpm (21), 16 700 cpm (27). Thus position of these large T_1 oligonucleotides relative to poly(A) end is in the order indicated by the associated radioactivities. T_1 oligonucleotides 11, 19, 20 and 28 have been further ordered after quantitation of their respective radioactivities in the 18-22S poly(A) RNA. To map T_1 oligonucleotides 17, 26 and 41, it was taken into account that they were consistently found less radioactive (30-40%) than the others, using control 35S RNA.

Is the mutant LA23 becoming transformation defective ? It has previously been reported that clonal isolates of RSV, when passaged in tissue culture under non-selective conditions, yield a mixed population containing both parental transforming and transformation defective (td) virus within a few generations (see ref. 4 and our own observations). The change from transforming to td virus derives from a deletion mutation that has been characterized in the case of RSV Pr-B by the loss of 3 large

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T_1 oligonucleotides located in the onc gene proximal to the poly (A) (4).

We have been looking for td mutants of RSV LA23 by performing the following experiments : secondary cultured cells were infected at low (about 0.1 PFU/cell) and high (about 10 PFU/cell) multiplicities, at permissive (35°C) and non-permissive (41°C) temperatures for cell-transformation. Cells were cultured for 5 days with daily medium changes and the 5th day medium was used to reinfect new secondary cultures. After three and 12 successive passages, virus grown under the four different conditions were purified and their 70S RNA was extracted and fingerprinted. The results are shown in Fig. 6. It is clear that none of the large T_1 oligonucleotide markers for the onc gene or for the other regions of LA23 are missing even after 12 successive passages of the virus infecting cells at a high multiplicity (Fig. 6c to f). This correlates well with the visual observation of the cells that looked completely transformed by the virus at the 12th passage at 35°C, but not at 41°C. One observes however the presence of additional minor spots in the fingerprint of LA23 RNA after 12 passages. On the basis of their relative migrations, these minor spots may correspond to large T_1 oligonucleotides found in 28S and 18S ribosomal RNA. Under similar conditions but using RSV pR-B RNA we have observed that the amount of T_1 oligonucleotides 5 and 6 (markers for the onc gene of Pr-B RNA) have decreased three fold after three successive passages, a fact which correlates with the appearance of td particles.

DISCUSSION

We report here a detailed analysis of the genome of the LA23 (subgroup B) T/S mutant for transformation of RSV; this analysis was undertaken in view of a number of interesting properties of this mutant :

(i) The use of this rapid shift transformation mutant in the study of virus-mediated cell transformation.

(ii) The high level of virus production of this mutant as compared with wild-type transforming strains of RSV.

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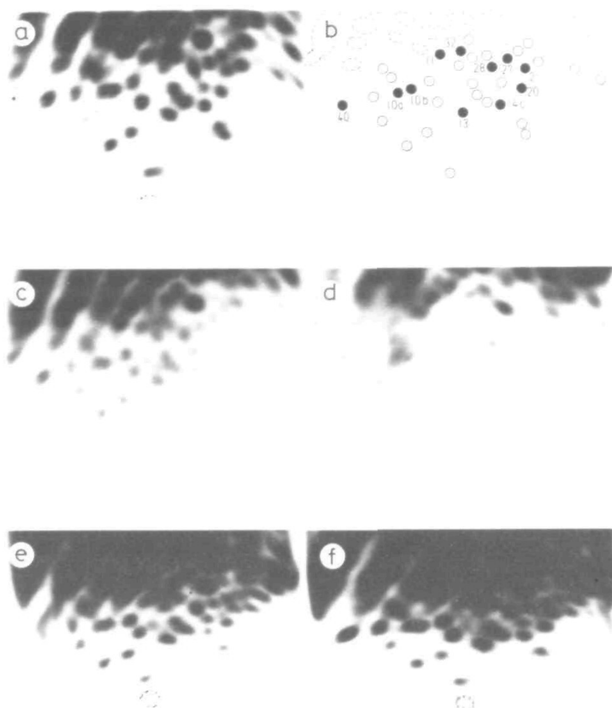

Figure 6

Presence of transformation defective (td) mutants of LA23 as analyzed by RNA fingerprint

LA23 was grown under 4 different conditions, at a multiplicity of about 0.1 PFU/cell at 35°C and 41°C, and multiplicity of about 10 PFU/cell at 35°C and 41°C. After 3 and 12 passages under these conditions, the virus was labelled with (^3H) uridine, collected, and 70S RNA purified as described in Methods.

70S RNA (about 0.6 µg) was extensively digested with T_1 RNase and the T_1 oligonucleotides were labelled at their 5' end with ($\gamma^{32}P$) ATP and T_4 polynucleotide kinase as described in Methods. Autoradiography was for 10 hr using Kodak XR5 film.

Control 70S RNA (a) and the schematic representation of the fingerprint indicating the T_1 oligonucleotides located in the onc gene (b). Fingerprint of RNA from virus grown after infection at a multiplicity of 10 PFU/cell, at 35°C (c, 3 passages) (e, 12 passages) or at 41°C (d, 3 passages) (f, 12 passages). Results obtained after infection at a multiplicity of 0.1 PFU/cell are identical.

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(iii) A remarkable stability of the onc gene sequences on high multiplicity passage of the virus, at least as far as concerns all large T_1-oligonucleotides in this gene.

The 38 large T_1-oligonucleotides separable on two-dimensional gel analysis have been ordered relative to the 3' poly(A) end of the RNA subunit. These oligonucleotides have been sequenced, resulting together with the 5' end sequence already reported (13) in a total of 750 nucleotides of the LA23 mutant that have been sequenced. A marker oligonucleotide of known sequence is thus expected statistically every 250 nucleotides along the subunit.

The entire mapping results and sequence determination have been performed using unlabelled genomic RNA, and labelling oligonucleotides after T_1-ribonuclease digestion with (γ ^{32}P) ATP and T_4 polynucleotide kinase. Under these conditions oligonucleotides 17, 26 and 41 were found to be incompletely labelled *in vitro* and this was taken into account in the mapping experiments (see figure 5 and table 2).

LA23 is derived from Pr-B by mutation(s) affecting the transforming (onc) gene (1). The onc gene has been located at the 3' end of the genome (4, 11) with its 5' end at about 3000 nucleotides from the poly(A) end (16, 17). The markers for the 3000 residues should thus be the eleven poly(A) proximal large T_1 oligonucleotides that are 11, 28, 20, 10b, 13b, 32, 40a, 10a, 14c, 21 and 27 (fig. 6 and table 2). Only 3 T_1 oligonucleotides are in common with the T_1 oligonucleotide markers in Pr-A or Pr-B RNA (table 3). This result means that the onc gene region of Pr-A or Pr-B RNA has been heavily mutated to give that of the mutant LA23.

We have observed a remarkable stability of all T_1-oligonucleotides in 70S RNA preparations of LA23, even when the virus was passaged twelve times successively at high and at low multiplicity of infection and at both the permissive and nonpermissive temperatures (fig. 6). These fingerprint analyses do not exclude the possibility that small deletions or point mutations, that would not affect the large T_1-oligonucleotides, have occurred in the region of the onc gene.

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Table 3. The T_1 oligonucleotide markers in the onc gene region of LA23 RNA and Pr-A, Pr-B or Pr-C.RNA

Number	Molar yields of pancreatic RNase digestion products	virus
27	A_2U, AU, 4C, 5U, G	LA23 [a]
21 [c]	A_2U, AC, 3C, 6U, G	
14c	A_4U, 2AU, 4C, 4U, G	
10a	3AC, AU, 5C, 2U, G	
40a	AG, 3AC, AU, 5C	
32	A_2C, $2A_2U$, 2C, 2U, G	
13b [d]	A_2C, A_2U, AC, 2AU, 3C, 4U, G	
10b	A_3U, 2AC, 7C, U, G	
20 [e]	A_2U, AU, 3C, 6U, G	
28	A_2U, AC, AU, 3C, 4U, G	
11	A_2C, AC, AU, 5C, 4U, G	
5	A_3U, 2AC, AU, 4C, 3U, G	Pr-A, Pr-B, Pr-C [b]
6	AG, 2AU, 5AC, 13C, 8U, G (mixture)	
14 [d]	A_2C, A_2U, AC, 2AU, 3C, 4U, G	
21 [c]	A_2U, AC, 2C, 6U, G	
26	2-3AU, 7U, G	
10	A_2C, AU, 2AC, 5C, 2U, G	
20 [e]	A_2U, AU, 3C, 7U, G	Pr-B only
111	A_2U, AU, 4C, 6U, G	Pr-A and Pr-C

(a) Starting from uniformly labelled (^{32}P) LA23 RNA, spots corresponding to the numbers in Figure 1 were eluted from the gel, digested with pancreatic RNase and analyzed by electrophoresis on DEAE - cellulose paper at pH 3.5. The values given are average of 3 determinations.

(b) Results are taken from references 4 and 14.

(c) (d) and (e) refer to the T_1 oligonucleotides that are common on the basis of their relative migation in a 2-dimensional polyacrylamide gel electrophoresis (fingerprint), and of their pancreatic RNase digestion products.

We are presently examining the potential of the LA23 mutant for elucidating the mechanism of td mutant formation in RSV stocks.

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