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High spontaneous mutation rate of Rous sarcoma virus demonstrated by direct sequencing of the RNA genome

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ABSTRACT

Direct and extensive sequencing of RSV RNA genome is reported. More than 10,000 nt* of the T_1 RNase resistant RSV RNA fragments (1) have been sequenced and shown to cover 3900 nt of RSV genome. The frequent sequence variations found indicate that RSV supports a very high incidence of spontaneous mutations in the course of replication, one very probable cause of the genetic diversity among the avian retroviruses. Sequences of the structured RSV RNAs allowed us also to precisely characterize the structured domains of the retroviral genome and show that the src gene is not structured.

INTRODUCTION

As already well documented and discussed (3) Rous Sarcoma virus (RSV) RNA can direct four different biological functions : reverse transcription, RNA splicing, translation and virion assembly. Because of the role of RSV RNA in these various processes we initiated a study of its structure as this was expected to provide informations about the molecular basis for the specificity of these functions (2).

We and others have previously shown that RSV 35S RNA has a highly ordered structure (2,4) and that the two subunits are intimately associated not only at their 5' ends (2,5) but also via numerous other specific interactions to form the 70S RNA complex found in mature virions (2,4,5). A large number of secondary structures along the 35 RNA subunit have been characterized with respect to their length and localization along the genome (1). Recently two structures of RSV RNA have been sequenced, i.e. the 5' untranslated region called leader sequence and the dimer linkage structure, and the role of the leader in initiation of translation and reverse

transcription, and that of the dimer linkage in virion assembly have been studied and discussed (6,7). Now all the other RNase resistant RNA fragments (structured RNAs) have been sequenced, which leads to the direct determination of more than 10,000 nt covering 3900 nt of RSV genome. In the course of this study we discovered a large number of sequence variations in RSV RNA as demonstrated by the sequence of individual RNA fragments mapping at the same site. These variations observed in RSV RNA sequence indicate that the virus supports a very high incidence of spontaneous mutations during its replication, a fact which probably accounts for the extensive genetic diversity in retroviruses.

MATERIALS AND METHODS

Cell and viruses. RSV Prague B-LA23 (t/s mutant in Src) given to us by J. Wyke; 8) was recloned and grown in secondary cultures of chicken embryo fibroblasts (9). The recloned virus grown in large amounts was found to retain all its capacity to transform cell at 35°C and not at 41°C. In addition T_1 oligonucleotides 8 and 41 specific for Src (10) were as abundant as others specific for Gag, Pol and Env (not shown). The size of Pr-B (LA23) 35S RNA was found to be 9-10 kb as for Pr-A 35S RNA (agarose gel electrophoresis). A td isolate of LA23 was found to be 0,5-1 kb shorter (J.-L. Darlix, unpublished results).

Isolation of virion nucleic acids. 70S and 35S RNA labelled with (^{3}H) uridine or (^{32}P) orthophosphate were purified as previously described (9).

Enzymatic degradation of RSV RNA with RNases III and H (11,12) or T_1 RNase was carried out as reported (2).

Purification of (^{32}P) RSV RNA fragments. Following partial enzymatic degradation of (^{32}P) 35S RNA with RNase III or RNase H or T_1 RNase, (^{32}P) RNA fragments were purified by 6%-12% polyacrylamide gel electrophoresis (PAGE) in 75 mM Tris-borate, 1 mM EDTA, 0,1% SDS. The components were detected by autoradiography, excised and eluted. Each RNA fragment was labelled in vitro using (^{32}P)ATP (3000 Ci/mmole) and T_4 polynucleotide kinase (Amersham, P.L. Biochemicals)

under conditions described before (9). 5'-^{32}P RNAs were purified as follows : 1) 2 dimensional PAGE, first dimension being 6%-10% polyacrylamide in 25 mM citric acid, 7 M urea, second dimension being 12% to 22% polyacrylamide in 75 mM Tris-borate, 1 mM EDTA, pH 8.3. After autoradiography radioactive spots were cut out and 5' ^{32}P RNAs eluted and further purified; 2) PAGE (6 to 20% polyacrylamide) in 7 M urea, 75 mM Tris-borate, 1 mM EDTA, pH 8,3. 5'-^{32}P RNA were recovered and sequenced.

Sequencing of RNA. We followed the method described by Donis-Keller et al. (13) to map adenines, guanines and pyrimidines and we used Physarum I and M RNases as well as Bacillus Cereus and Neurospora crassa nucleases to distinguish between uridines and cytidines (14). The partial cleavage products were separated on 6-12% PAGE in 7 M urea, 75 mM Tris-borate, 1 mM EDTA, pH 8,3.

RESULTS

A method to purify unique RNA species

Polyacrylamide gel electrophoresis (PAGE) of RNA under various ionic conditions was shown by Fiers and coworkers (16, 17) to be a method of choice to purify RNA species and more precisely T_1 RNase resistant fragments (the structured RNAs) of MS2 RNA. We used this method, adding modifications and a final step (PAGE in 7 M urea) to obtain and further sequence small RNAs from Chlamydomonas reinhardii (15).

RSV 35S RNA was first partially digested with T_1 RNase and families of structured RNA fragments were purified by polyacrylamide gel electrophoresis (PAGE) in non denaturing conditions and further analysed by T_1-fingerprinting as reported before (1,9,16). To obtain unique RNA species, each family of structured fragments was first 5'-labelled with ^{32}P and RNA purified by acid-urea and neutral PAGE followed by electrophoresis in 7 M urea at pH 8.3. As an example, the purification of two families of structured RSV RNA fragments is shown in fig. 1a (2 dimensional PAGE) and 1b (PAGE in 7 M urea at pH 8,3). Each of the structured RNA fragments 9 and 10 correspond to a unique band upon PAGE in non denaturing

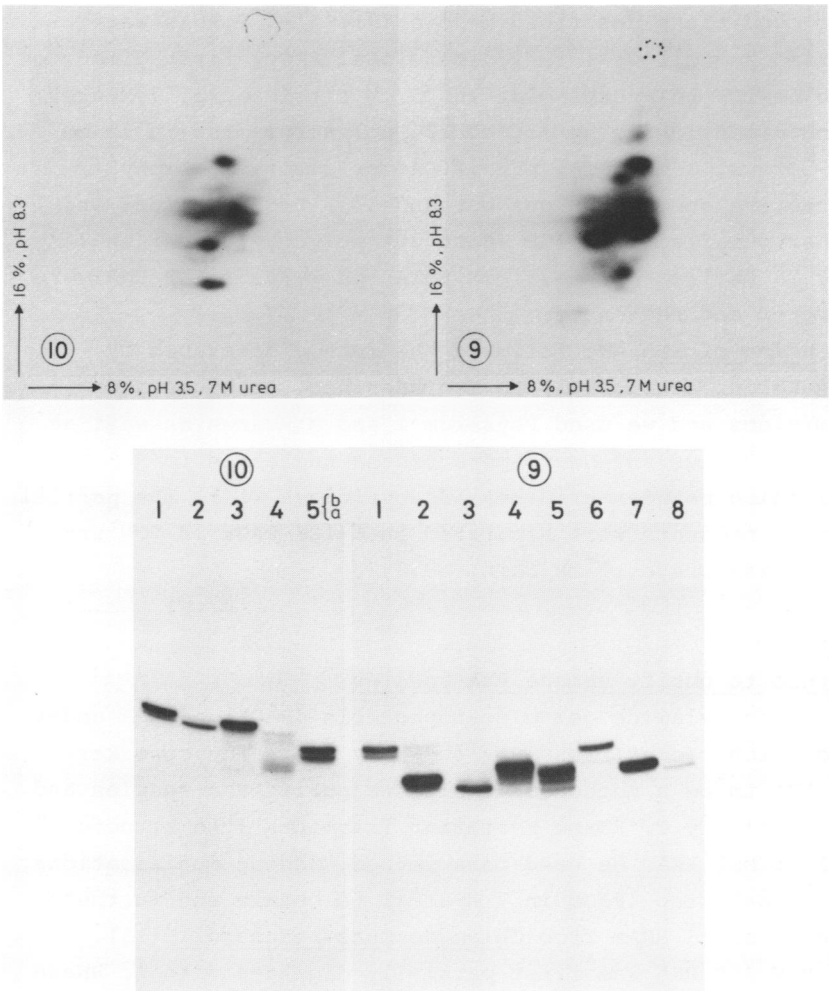


FIGURE 1 : Purification of families of RSV structured RNAs

Structured RNAs 9 and 10 were obtained as reported before (1). 5'-end (P^{32}) labelling of RNAs was as described in materials and methods.

a) 2d PAGE in 25 mM citric acid and 7 M urea (0,15 x 1,5 x 40 cm) and then in 75 mM Tris-borate, 1 mM EDTA pH 8.3 (0.15 x 30 x 40 cm). Exposure was for 10 min using Kodak XS films. Circle at the top refers to xylene cyanol. Spots were cut out and radioactive RNAs eluted.

b) 12% PAGE in 8 M urea and 75 mM Tris-borate, 1 mM EDTA pH8,3 (0,03 x 30 x 40 cm). Radioactive RNA bands were cut out and numbered as indicated on top of the figure. After elution in presence of 10 µg of purified yeast tRNA, followed by three ethanol precipitations, 5'(P^{32}) RNAs were sequenced as described in materials and methods.

conditions (see fig. 1 and 2 in ref. 1).

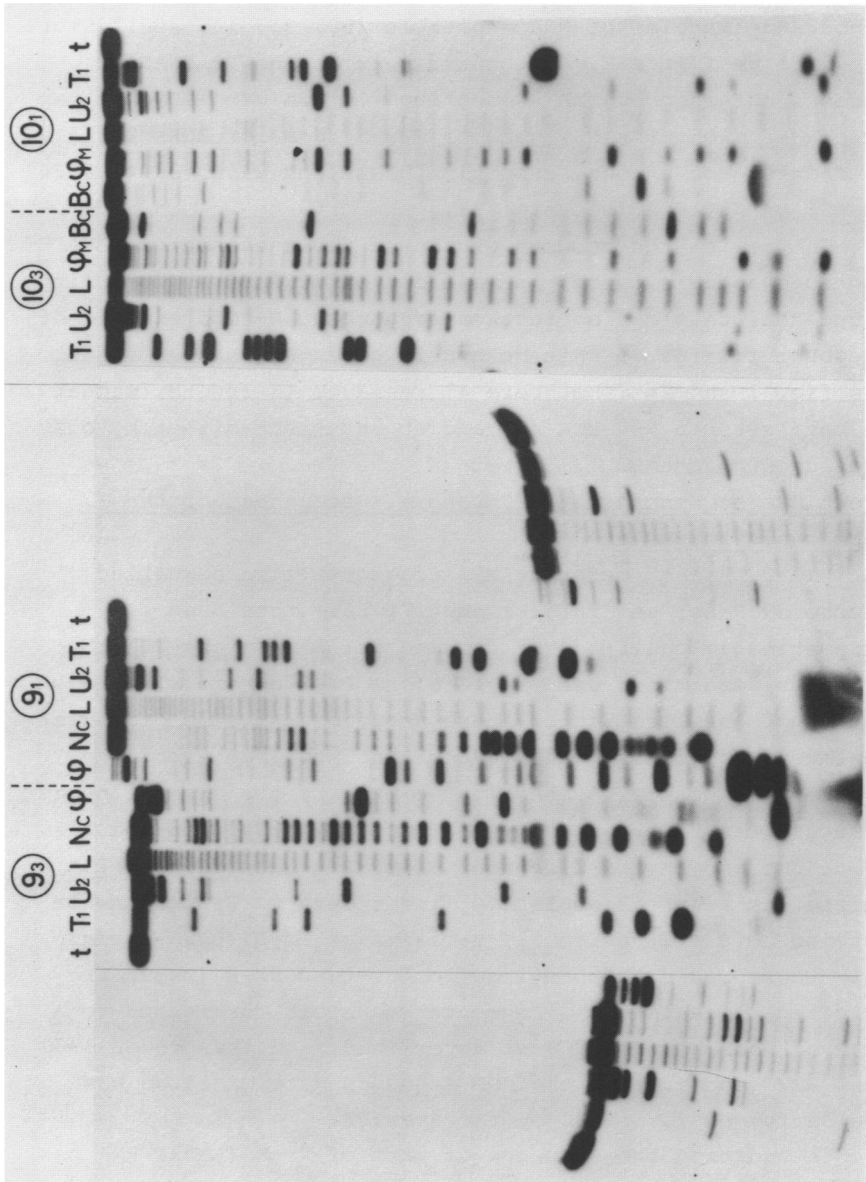
All the RNAs numbered in figure 1b (except RNA 10_4) were successfully sequenced using the rapid enzymatic sequencing method and examples of RNA sequences (9_1, 9_3, 10_1 and 10_3) are given in figure 2. By comparison with the Pr-C RNA sequence obtained by DNA sequencing (10) we were able to map these RNAs along the RSV genome. The 5' ends of RNAs 9_1, 9_3, 10_1 and 10_3 are at the respective positions 2919, 3824, 6024 and 430 on RSV RNA (see fig. 3).

In view of the results presented above the method described appears to be a simple and efficient way to purify unique RNAs that can be further sequenced. A good example of the possibilities of this method is given by RNAs 9_2 and 9_3 (see fig. 1 and 2) : both are 47 residues in length, map at the same site on RSV RNA (5' end at 3824) but differ by only 3 residues.

Direct RNA sequencing of RSV genome : sequences of the T_1 RNase resistant RNAs

All T_1 RNase resistant RNA fragments (the so-called structured RNAs; see refs. 1 and 17) have thus been systematically purified and sequenced : 160 unique RNA sequences determined and mapped along Pr-C genome (10). A summary of the location of these sequenced RNAs is given in the legend to figure 3 showing that a large number of the structured RNAs are located in the leader, the dimer linkage structure, at the 5' and 3' ends of Gag and Pol, at the 3' end of Env and in the C region. All RNA sequences are reported in figure 3 where top line refers to Pr-C sequence (10) and bottom lines to all variants of Pr-B (LA23) found (only those residues changing from Pr-C to Pr-B (LA23) are indicated). The 160 RNA fragments sequenced correspond to a total of more than 10,000 nt determined, and they cover 3900 nt of RSV genome. Thus the RNA sequence determination has been performed 2,5 times on the average.

In addition the sequence of part of Pr-B (LA23) RNA has been determined independently and the results confirmed the data reported in figure 3. Results include sequences of 43 unique T_1 oligonucleotides (9), of RNA fragments generated


(10₁)
T₁ U₂ L φM Bc Bc φM L U₂ T₁ t
(10₃)
(9₁)
t T₁ U₂ L Nc φ Nc L U₂ T₁ t
(9₃)

upon cleavage of RSV 35S RNA by E.coli RNase H in presence of d(TCCAT) which correspond to the 5' untranslated region or leader (7,12) and to positions 2125-2180 (not shown), and of cDNA transcripts at positions 1-101, 2000-2140, 2620-2770 and 2800-2910 (12). Finally the segment of $tRNA^{trp}$ initiator hydrogen bonded to RSV RNA was found to comigrate with structured RNAs of the leader in PAGE under non-denaturing conditions. Sequence of this 5'-P^{32} RNA segment was found to be GAAmAUCACGUCGGGGUCACCA showing the presence of an additional A residue as compared to the known sequence of $tRNA^{trp}$ (18).

High mutation rate and frequent amino-acid variation

Upon purification and sequencing of the T_1 RNase resistant RNA fragments, more than one fragment was found for a given sequence in most cases. The sequence variations are thus based on independent sequence analyses (bottom lines in figure 3) and amount to 620 base changes (including insertions and deletions) in the 3900 residues determined. This high mutation rate of RSV RNA is correlated with frequent amino-acid changes in the viral proteins. One example is given in figure 4 where the RNA sequence of the 3' end of Env (6714-6862, see fig. 3) has been translated into amino-acids according to the reading frame given by the work of Schwartz et al. (10). 32 nucleotide variations are observed in the three subspecies of Pr-B (LA23) corresponding to 20 amino-acid changes in the Env protein Gp 37. Similarly 24 nucleotide variations are observed at the 3' end of P19 coding sequence (pos. 806-963, see fig. 3) corresponding to 16 amino-acid changes. More generally a computer analysis of

FIGURE 2 : Sequence of RSV structured RNAs

Unique RNAs were obtained as described in materials and methods and figure 1. Partial nuclease digestions were as follows : T_1 is T_1 RNase in 7 M urea 25 mM citrate pH 4,5, U_2 is U_2 RNase in 7 M urea 20 mM citric acid pH 3,5, L is ladder (20 min at 100°C in 90% formamide), P_M is physarum M RNase in 7 M urea 20 mM citrate pH4,5, N_C is Neurospora crassa nuclease in 7 M urea 20 mM citrate pH4,5 (partial digests are migrating with a difference of -1 and thus sequence should be read in consequence). Bc is Bacillus cereus nuclease in 20 mM Tris-citrate pH 7,5. Partial digests were run on 12% PAGE.

the mutations indicates that more than 50% of the spontaneous mutations result in amino-acid changes and then modify the viral proteins. Such protein modifications are well illustrated by an observation made with the LA23 isolate of RSV Pr-B we use. This isolate grows very well (250 µg of 70S RNA per liter of culture medium and $\geqslant 10^7$ FFU per ml), is stable as compared to other isolates of Prague or of Schmidt Rupin (2, 19) but shows an abnormal slow maturation of viral protein P19. Even with 3-6 h harvest virus about 50% of P19 is found in P22 - the precursor to both P19 and P10 (10,21), which indicates that the proteolytic cleavage of the p19-P10 bond is inefficient. In fact the P19-P10 bond located at positions 905-910 on Pr-C sequence (10) is heavily mutated in Pr-B (LA23) RNA (fig. 3) - the deduced amino-acid sequence around the P19-P10 bond is :

P19-Pro Tyr] Val Gly [Ser Gly-P10 in Pr-C (10) and

P19-Pro Tyr] Val Pro [$^{\text{Met}}_{\text{Thr}}$)($^{\text{Gly}}_{\text{Thr}}$)-P10 in the three variants of Pr-B(LA23). Consequently substitution of a glycine by a proline in the P19-P10 bond renders this site poorly cleaved by viral protein P15. This mutation does not affect both the production of large amounts of virions and the infectivity of the virus (see above).

DISCUSSION

Purification and sequencing of unique RNA species

Principles of the purification method used here are basically those developed for sequencing bacteriophage MS2 RNA (17). An additional purification step (PAGE in 8,3 M urea) was found to be necessary in order to obtain unique RNA sequences 35-200 residues in length. This method proved to be highly resolutive since pure subspecies of RSV structured RNAs and of other RNAs (15) have been obtained and sequenced although nucleotide differences were sometimes not more than 2 residues per 100 nucleotides (see fig. 3). RNA sequencing was performed using the partial enzymatic degradation of ^{32}P end labelled RNA since it is rapid, convenient (no need of special chemicals) and it gives clear sequence data. However when structured RNAs rich in G+A clusters are sequenced,

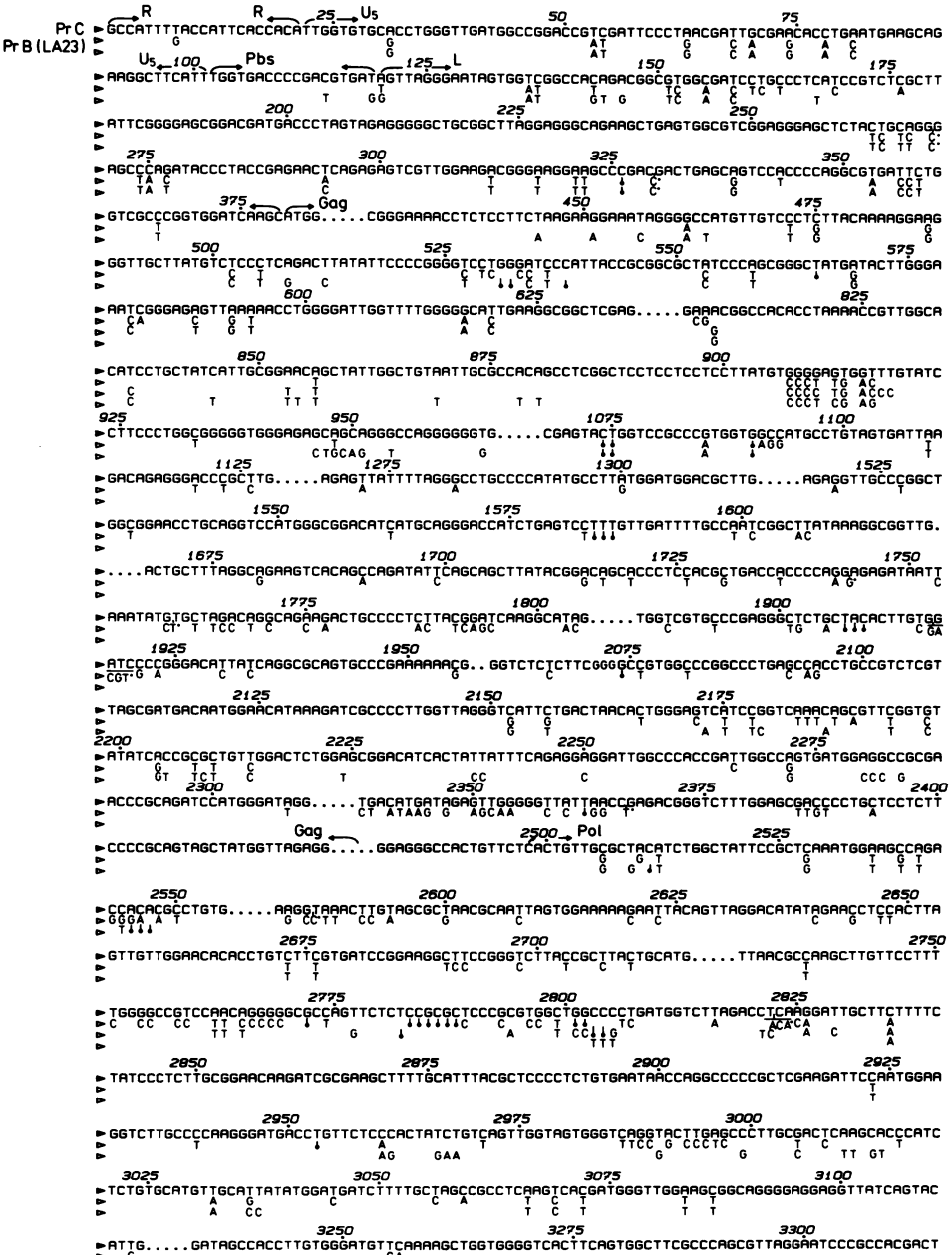
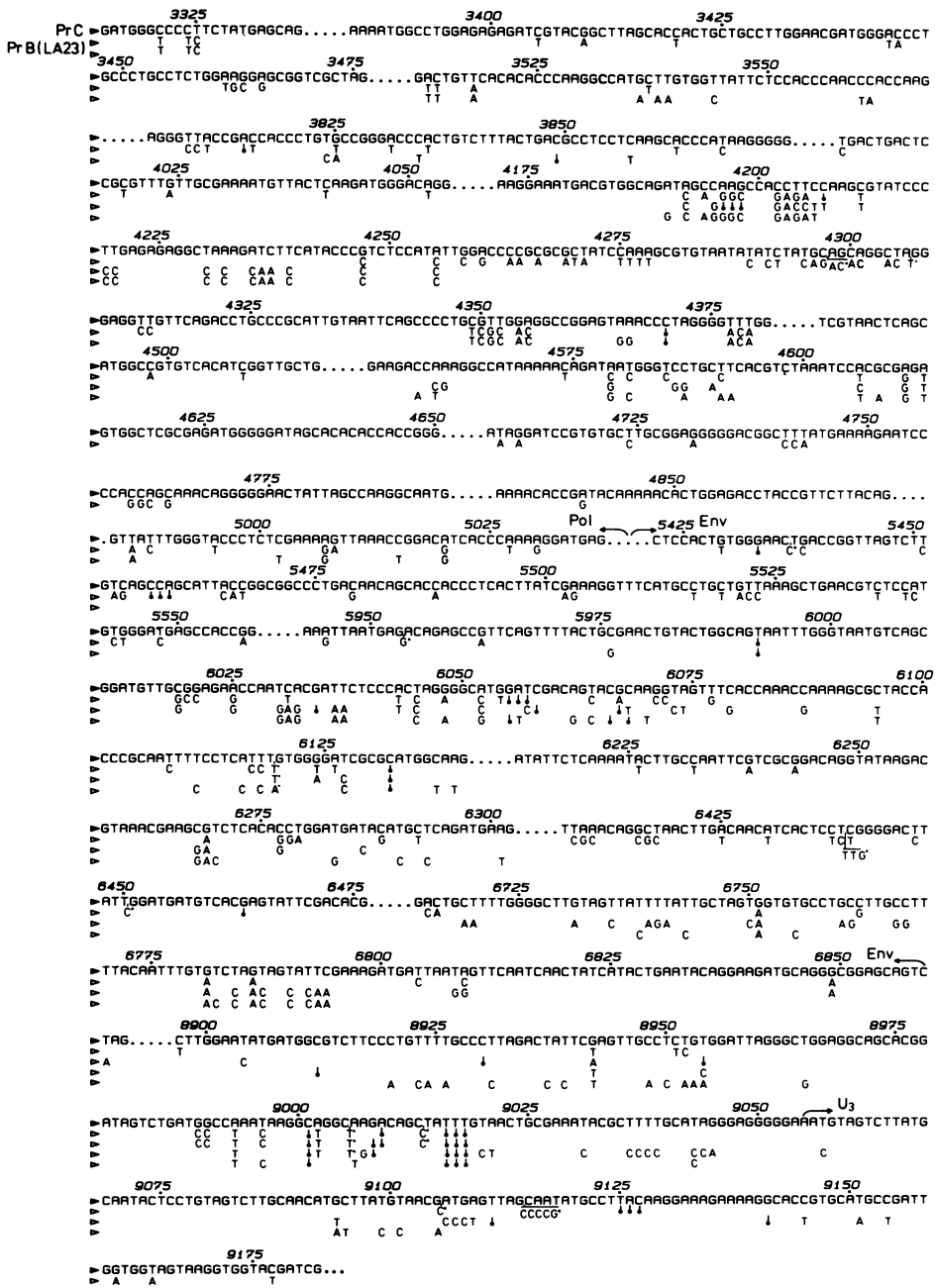


Figure 3


PrC
PrB(LA23)
Pol
Env
Env
U3

polyacrylamide gel electrophoresis in urea were carried out at high temperature to improve resolution of the G's (see fig. 3; pos. 300-325, 940-980).

Structured domains of the viral 35S RNA

We have previously studied the secondary structure of RSV RNA by means of specific nucleases (2). In a next step we have mapped the structured domains of the viral RNA upon characterization of T_1 RNase resistant RNA fragments (1). Recently the leader and the dimer linkage structure have been sequenced and a probable secondary structure proposed for both (6,7). Present sequence data of all T_1 RNase resistant fragments allow a precise mapping of the structured domains of RSV RNA (see figure 3) and reveal also that the U_3 region is structured a fact which was not detected before. In summary, the leader, the 5' and 3' ends of Gag and Pol, the 3' end of Env and the U_3 region exhibit stable secondary structures. On the other hand no stable RNA structure has been observed in the Pol-Env and Env-Src junctions as well as in the Src gene. The absence of folded sequences in the Src gene (for identification of Src in Pr-B (LA23) see Materials and Methods) argues in favor of recombination events at the RNA level since the v.Src has lost the introns present in the c.Src (22-24) and part of the introns present in hn RNA's are known to correspond to stable RNA structures (25,26).

DNA and RNA sequencing of the RSV genome : a comparison

Schwartz et al. (10) have determined the complete

FIGURE 3 : Nucleotide sequence of the structured domains of RSV RNA

RNA sequencing was as described in materials and methods (e.g. see fig. 1 and 2). Structured sequences located along RSV genome were distributed as follows : number of RNA fragments per 0,3 Kbase from 5' to 3' is 10 (leader), 9 and 8 (P19 coding sequence), 9,3,4 (P27 coding sequence), 1 (P12 coding sequence), 7 (3' of Gag), 10 (5' of Pol), 12, 4, 5, 4, 1, 8, 12 and 7 (3' of Pol), 0 (Pol-Env junction), 2, 2, 13, 5, 10 (3' of Env), 0 for both Env-Src junction and Src (pos 6900-8700) and 5 and 9 (C region). Top line refers to the DNA sequencing of cloned Pr-C (10) bottom lines correspond to Pr-B (LA23) RNA sequences where only those residues specific for Pr-B (LA23) RNA are indicated. ^{o} Deletions are indicated by (e.g. pos 326) and insertions by N (e.g. pos 329-330). Nucleotide number is according to Pr-C RNA.

```
                                        (Leu)
GlyLeuLeuGlyLeuValValIleLeuLeuLeuValValCysLeuProCysLeuLeuGln-
Phe
   Met                                                 Arg
      Asn       IleAla   Glu       Ala               AlaTrpVal
                     Thr                 Arg

ValSerSerSerIleArgLysMetIleAsnSerSerIleAsnTyrHisThrGluTyrArg-
Lys
      Asn                 Thr   Arg
      Asn    Thr             LysGly
   ProAsn    Thr

MetGlnGlyGlyAlaValterm
      Asp
      Asp          Lys
```

FIGURE 4 : Deduced amino acid variability at the COOH terminus of RSV Env protein Gp37

The complete amino-acid sequence is taken from the work of Schwartz et al. (10) with Pr-C. The other lines refer to Pr-B (LA23) and only those residues that differ from Pr-C are indicated. Residue in parentheses (Leu) indicates two base variations observed in Pr-C sequence (10).

nucleotide sequence of Pr-C RNA by means of DNA sequencing. We have used the direct sequencing approach to determine part of Pr-B (LA23) RNA sequence. The LA23 mutant of Pr-B used here was derived from Pr-A (LA23) crossed with RAV-2 (8); T_1 fingerprint analyses of Pr-A, Pr-A (LA23) and Pr-B (LA23) are indistiguishable (J.-L. Darlix, unpublished results) but differ considerably from that of the original Pr-B strain that was first analysed and proved later to be unstable (2, 19). In figure 3 the sequence of Pr-C DNA clone pATV-8 (10) is given as a reference to our RNA sequences of Pr-B (LA23) and it appears that sequences of these two isolates of RSV-Prague having a different subgroup differ noticeably if only the variations common to all RNA subspecies are considered (171 base changes), or greatly if all RNA variants are taken into account (781 base changes). This very last point emphasizes the fact that direct RNA sequencing can detect numerous variants of RSV RNA (see fig. 3) which are ignored completely or to a great extend upon sequencing cloned DNA or cDNAs, respectively (10).

Nature of the mutations

Single mutations are frequent and with no obvious effect on viral expression unless they introduce a terminator codon

(e.g. 2774, 2971) or change the reading frame (e.g. 531, 532 and 538). However if such alterations were introduced into the genome, the presence of non mutated sequence subspecies (e.g. 2730-3325) would lead to the synthesis of the viral proteins and to the packaging of both mutated and non mutated RNA sequences. Other mutations are insertion of one nt followed by deletion of one nt to maintain the reading frame (e.g. 2362-2366; 5953-5992), deletions of exactly 3 bases (e.g. 1584, 1907, 2545, etc.) or of 2 bases and then of one to restore the reading frame (e.g. 531, 1074), insertions of exactly 3 bases (e.g. 2822, 6438) or 2 bases and then one (e.g. 1743-1760). Larger deletions or insertions appear to take place between repeats of 5 or more bases as it has been observed before (27) (e.g. 1907-1961 ACACT; 2783-2800 CCGCGCC). Nevertheless sites of importance for the functions of RSV RNA in reverse transcription, translation and particle formation do not appear to support a high incidence of mutations : (i) the R sequence following the Cap, the tRNA primer binding site at 101-118 and the polypurine track at 9048-9056 as well as the 5' end of the U_3 region at 9057-9086 (3), (ii) the strong ribosome binding site and its interacting sequence at 1-50 and 340-380 (28) as well as the 5' ends of Gag and Pol, (iii) the P19 binding sites very probably implicated in the dimer linkage structure and in virion assembly at nt 430, 510, 891 and 2393 (6).

High spontaneous mutation rate and genetic diversity of retroviruses

As pointed out by Temin (29) there is extensive genetic diversity among the avian and murine leukemia and sarcoma viruses. Genetic diversity has been found in all viral proteins and also in the ability of the retroviruses to infect heterologous cells, to form neoplastic tumors and in the types of tumors formed.

Several genetic studies indicated that there is a high frequency of spontaneous variation in avian and murine leukemia and sarcoma viruses (30-33). Similarly biochemical studies have shown that RSV and SNV support a high incidence of spontaneous mutations in the course of replication, and

that the mutations occur in all viral genes (2,19,29,34). The present RNA sequencing data on RSV RNA are in total agreement with the genetic and biochemical data, and directly demonstrate that frequent spontaneous mutations occur in Gag, Pol, and Env genes and also in the 5' leader, and in the 3' U_3 region believed to define the exogenous or endogenous phenotype of avian retrovirus (35). Thus the high incidence of spontaneous mutations in the viral genome accompanied with frequent changes in the viral proteins, and this coupled with host selection (homologous or heterologous host cell or animal), should be regarded as one of the biochemical basis of the extensive genetic diversity among the avian and murine leukemia and sarcoma viruses. Presumably this high frequency of sequence variations is due to the infidelity of reverse transcription (36), although infidelity of RNA synthesis is also possible. The second and additional mechanism susceptible to explain the genetic diversity of retroviruses is the recombination between viral sequences and also between viral and normal cellular sequences (32,35,37).

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Abbreviations used : nt, nucleotide; RSV, Rous Sarcoma virus

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