

RNA Sequencing Provides Evidence for Allelism of Determinants of the N-, B- or NB-Tropism of Murine Leukemia Viruses

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Summary

Previous genetic and biochemical studies identified three large RNAase T1-resistant oligonucleotides, each associated with either the N-, B- or NB-tropism of murine C-type viruses of BALB/c origin. These oligonucleotides were shown to lie in the 5' third of the oligonucleotide maps of their respective viruses. We sequenced the three oligonucleotides and found that they share a 10 base sequence. Together these observations provide good evidence that the determinants of N-, B- or NB-tropism monitored by the three oligonucleotides are allelic.

The oligonucleotides associated with N- and B-tropism differ in sequence at four of sixteen nucleotides, while the B- and NB-tropism-associated oligonucleotides differ in sequence by only one base out of sixteen. These results are consistent with the possibilities that B-tropic viruses may arise from N-tropic viruses by recombination, while NB-tropic viruses may arise from B-tropic virus by mutation.

An unexplained finding was that a 10 base sequence present in the oligonucleotide associated with N-tropism is also found in the 3' third of the genomes of the N-, B- and NB-tropic viruses studied.

Introduction

Several lines of investigation have been directed toward characterizing and identifying the viral gene(s) that determine the N-, B- or NB-tropism of murine leukemia viruses (MuLV). Studies involving mixed infections with N- and B-tropic viruses (Rein et al., 1976; Ishimoto, Hartley and Rowe, 1978) or with MSV and N- or B-tropic viruses (Bassin et al., 1975) revealed that a determinant of the N- or B-tropism of murine leukemia viruses is present in virions and can participate in phenotypic mixing. It is simplest to imagine that this determinant, designated the "target" of the Fv-1 gene by Rein et al. (1976), is a protein. In genetic and biochemical studies, analysis of the virion proteins of an N- and

a B-tropic virus of BALB/c, of sixteen recombinants between these viruses and of eight NB-tropic viruses derived from the B-tropic virus suggested that p30 (the major internal virion protein of MuLVs), or possibly the product of a gene closely linked to p30 coding sequences, might be involved in determining the N- or B-tropism of MuLVs and might therefore be the target described by Rein et al. (1976) (Hopkins, Schindler and Hynes, 1977; Schindler, Hynes and Hopkins, 1977). In addition, analysis of the genomes of these same BALB/c viruses and their derivatives by RNAase T1 fingerprinting led to the identification of three large RNAase T1-resistant oligonucleotides that are associated with N-, B- or NB-tropism (Faller and Hopkins, 1977a, 1978b).

The three tropism oligonucleotides, defined by their electrophoretic mobilities and their secondary digestion products, were identified as follows. Five NB-tropic viruses derived from B-tropic virus of BALB/c acquire a common new T1 oligonucleotide, here designated the NB oligonucleotide, and all lose one and the same B virus-specific oligonucleotide, designated the B oligonucleotide (Faller and Hopkins, 1977a); all (sixteen) N-tropic recombinants inherit one N virus-specific oligonucleotide, the N oligonucleotide, and none inherits the B oligonucleotide that is lost when NB-tropic viruses are derived from the B virus (Faller and Hopkins, 1978b). The three tropism oligonucleotides lie in the 5' third of the oligonucleotide maps of N-, B- and NB-tropic viruses (Faller and Hopkins, 1978a). Partial sequence analysis of the tropism-associated oligonucleotides suggested that the B and NB oligonucleotides might be related by a single base change (Faller and Hopkins, 1977a); however, a relationship between the N and B or NB oligonucleotides was not obvious from these studies (Faller and Hopkins, 1978a).

Although the association of the N oligonucleotide with a determinant of tropism was only one of genetic linkage, available data are consistent with the possibility that NB-tropic viruses may arise from B-tropic viruses by mutation (see Lilly and Pincus, 1973; Hopkins, Traktman and Whalen, 1976) and thus that the B and NB oligonucleotides might lie in sequences which determine the tropism of MuLVs. Thus it was of interest to sequence the tropism-associated oligonucleotides for three reasons: to confirm the relationship between the B and NB oligonucleotides suggested by partial sequence analysis; to determine whether the N oligonucleotide was related to the B and NB oligonucleotides and thus whether a determinant of N-tropism might be allelic to a determinant of B- and NB-tropism; and to obtain the sequence of a region of the genome that is at least genetically linked to,

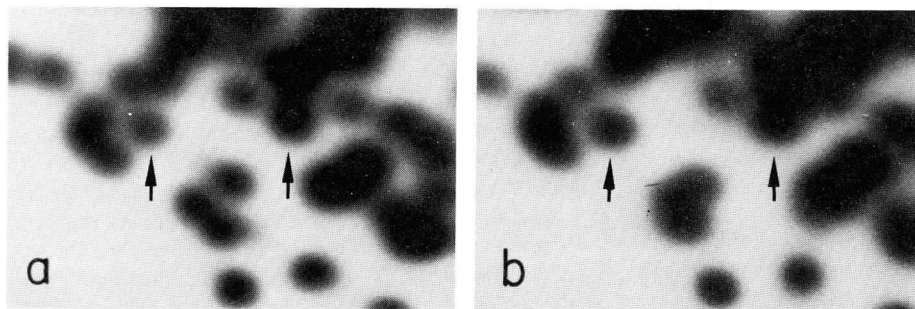


Figure 1. Corresponding Regions of RNAase T1 Fingerprints of LP-B Viral RNA Labeled with ^{32}P (a) in Vivo or (b) in Vitro. Black arrows indicate oligonucleotide 12 (left-hand arrow in each picture) or the mixture of oligonucleotides B+35.

but may actually comprise, a (the) determinant of viral host range.

Source of Oligonucleotides Whose Sequences Were Determined

An obstacle to sequencing the N and B oligonucleotides arose from the fact that in fingerprints of the N- and B-tropic BALB/c viruses that have been studied, each of these oligonucleotides co-migrates with a second oligonucleotide designated 12 and 35, respectively (Faller and Hopkins, 1977a, 1977b). Oligonucleotides 12 and 35 are also present in NB-tropic viruses (Faller and Hopkins, 1977a) and lie in the 3' third of the oligonucleotide maps of N-, B- and NB-tropic viruses (Faller and Hopkins, 1978a). Previous attempts to separate physically the N and B oligonucleotides from oligonucleotides 12 and 35, respectively, had failed. It was possible, however, to obtain the N oligonucleotide free of oligonucleotide 12 for the following reason: Akv, the N-tropic ecotropic virus of AKR mice (Rowe, 1972; Chattopadhyay et al., 1975), possesses both the N and 12 oligonucleotides found in the BALB/c N-tropic virus (Rommelaere, Faller and Hopkins, 1977); MCF viruses, which are recombinants between Akv and an unidentified xenotropic virus (Hartley et al., 1977), are N-tropic and possess the N oligonucleotide; however, one isolate, MCF V2-34, has lost oligonucleotide 12, presumably as the result of a recombination event in which the region of the genome containing oligonucleotide 12 was replaced with the corresponding region of a xenotropic virus genome (Rommelaere, Faller and Hopkins, 1978). In this virus, therefore, the N oligonucleotide appears alone in a T1 fingerprint.

Since all of the viruses studied so far that possess the B oligonucleotide also possess oligonucleotide 35, it was not possible to obtain the B oligonucleotide free of oligonucleotide 35. However, since oligonucleotide 35 can be obtained alone from fingerprints of N- or NB-tropic viruses, it was possible to deduce the sequence of the B oligonucleotide by subtracting the sequence of

oligonucleotide 35 from the sequence of a mixture of the B and 35 oligonucleotides.

Sequencing the Oligonucleotides

Oligonucleotides in an RNAase T1 digest of the appropriate viral RNA were labeled at their 5' ends with ^{32}P using polynucleotide kinase, and a two-dimensional gel electrophoretic fingerprint of the labeled digest was prepared. Figure 1 shows the relevant region of a fingerprint obtained by this method (Figure 1b) and, for comparison, the corresponding region from a T1 fingerprint of the same viral RNA labeled in vivo (Figure 1a) as previously described (Faller and Hopkins, 1977a). In vitro labeled oligonucleotides were removed from the second dimension fingerprinting gels and their sequences were determined by two methods, that of Silberklang, Gillum and RajBhandary (1977) and that of Donis-Keller, Maxam and Gilbert (1977). (Oligonucleotides N and 35 and the mixture B+35 were further purified before sequencing by banding on 20% acrylamide gels.) Typical results of both sequencing methods for the N and 12 oligonucleotides are shown in Figures 2 and 3, and for the NB, 35 and mixture of B+35 oligonucleotides in Figures 4 and 5. The sequences of the three tropism-associated oligonucleotides deduced from this type of analysis are shown in Figure 6. The sequences of oligonucleotides 12 and 35 are shown in Figure 7 adjacent to the sequences of the N and B oligonucleotides, respectively, with which they co-migrate.

The deduction of the sequence of the B oligonucleotide, obtained by subtraction, deserves comment. The three nucleotides adjacent to the terminal G in this oligonucleotide could not be unambiguously identified by homochromatography of the digestion products of the mixture of oligonucleotides B+35 (Figure 4). However, on the sequencing gels, by comparing the patterns obtained for oligonucleotide 35 with those obtained for a mixture of oligonucleotides B+35, it was possible to identify the bands belonging to each oligonucleotide in

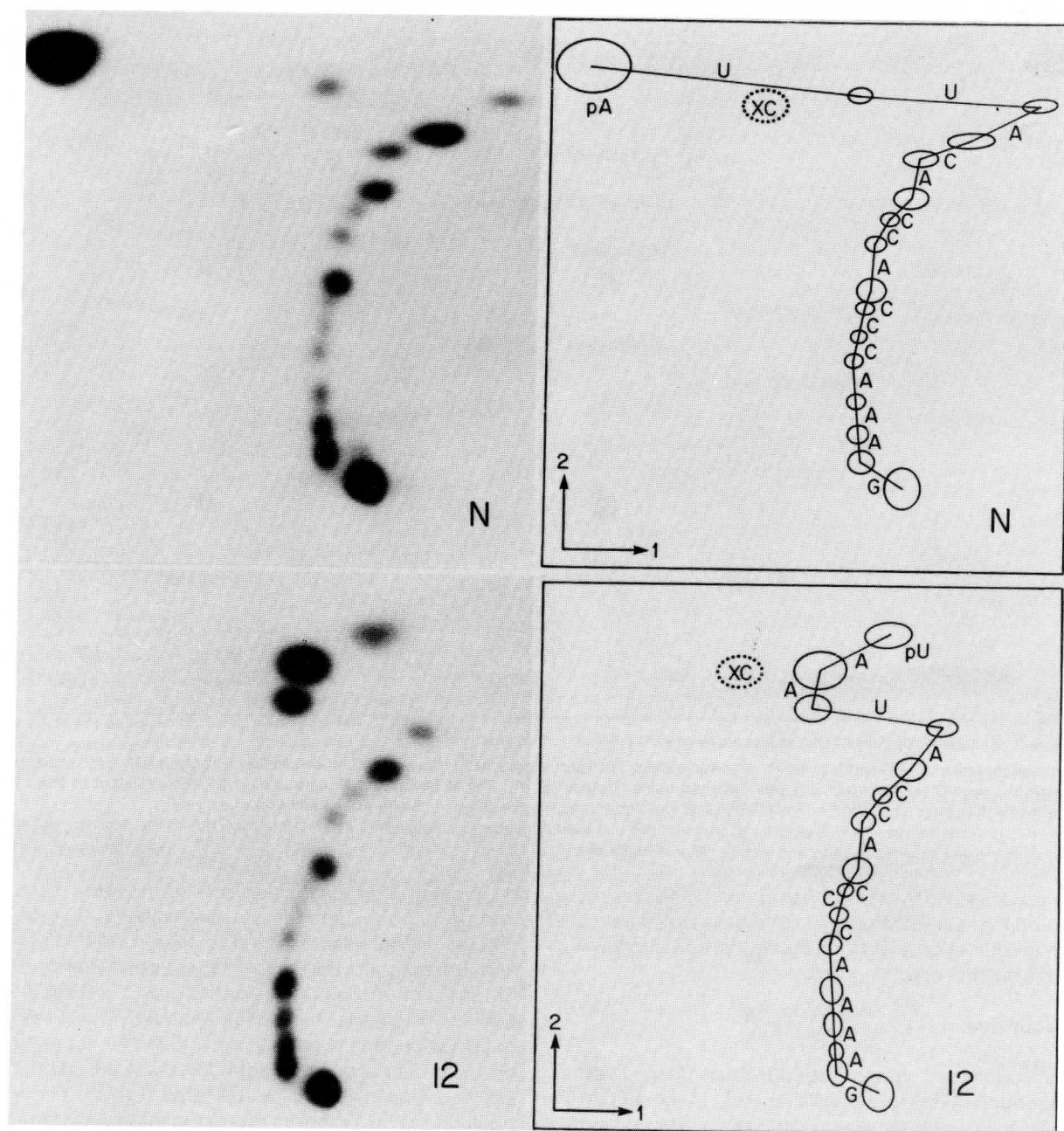


Figure 2. Sequence Analysis of Oligonucleotides N and 12

Autoradiograms and corresponding diagrams of the products of partial nuclease P1 digestion of 5'-³²P-end-labeled oligonucleotides after electrophoresis (direction indicated by arrow 1) and homochromatography (arrow 2). (XC) indicates position of dye marker, xylene cyanol.

this mixture and thus to obtain the sequence of the B oligonucleotide (Figure 5). The resulting sequence agrees well with the previously reported products of pancreatic RNAase digestion, deduced by subtraction, for the B oligonucleotide (Fallen and Hopkins, 1978a). The validity of the subtraction method was further substantiated by showing that the sequences of the N and 12 oligonucleotides could be obtained on sequencing gels from a

mixture of oligonucleotides N+12 (data not shown).

An unexpected observation was the failure of RNAase U2 to cleave the terminal A-G_{OH} sequence in all five T1 oligonucleotides analyzed (Figures 3 and 5). Possible explanations for this finding are that RNAase U2 requires a 3' terminal phosphate or longer base sequences at the 3' terminus or, less probably, that the adenine residues in all five

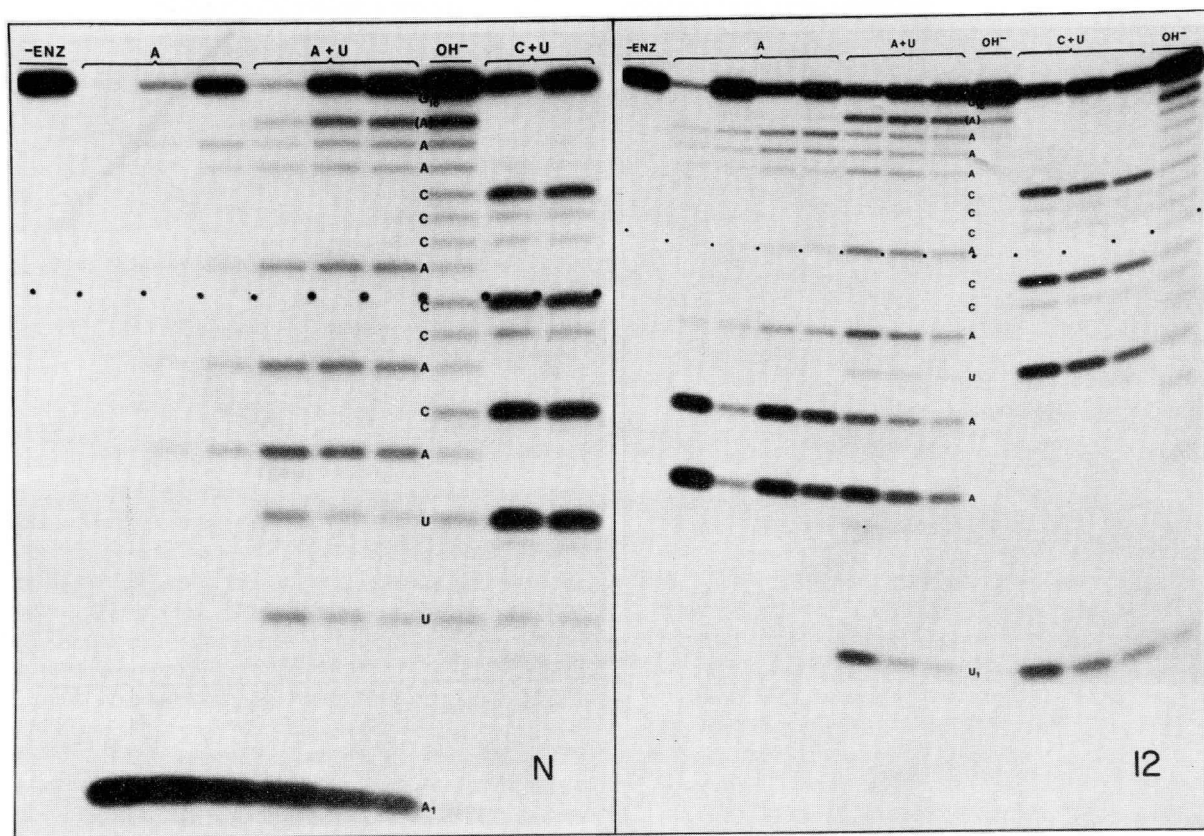


Figure 3. Sequence Analysis of Oligonucleotides N and 12

Autoradiograms of 5'-³²P-end-labeled T1 oligonucleotides after partial enzymatic cleavage or limited alkaline hydrolysis (OH⁻) followed by electrophoresis. Cleavage specificity was obtained using RNAase U2 (A), Phy M (A+U) and B. cereus (C+U). (-ENZ) aliquot of the T1 oligonucleotide after incubation in Phy M and U2 buffer under reaction conditions but without the addition of enzyme.

The larger gap between the full-length oligonucleotides and their derivatives lacking a terminal G is probably due to the retarding effect of guanine residues on electrophoretic mobility (Donis-Keller et al., 1977).

oligonucleotides are modified in such a way as to inhibit RNAase U2 cleavage. (We have no evidence for modification.) Further studies are in progress to clarify this point.

Discussion

We sequenced three large RNAase T1-resistant oligonucleotides previously shown to be genetically associated with either N-, B- or NB-tropism. The three oligonucleotides share a sequence 10 bases long, while the B and NB oligonucleotides share a 15 base sequence and differ by only one nucleotide. T1 oligonucleotide mapping had previously placed the three tropism oligonucleotides in the 5' third of the T1 oligonucleotide maps of their respective viruses (Faller and Hopkins, 1978a). This fact, together with the sequence data, would seem to provide good evidence for the allelism of the determinants of N-, B- or NB-tropism monitored by these oligonucleotides.

We also sequenced two T1 oligonucleotides,

designated 12 and 35, that co-migrate with the N and B oligonucleotides, respectively, in two-dimensional gel electrophoretic fingerprints of N- and B-tropic viruses. Oligonucleotides 12 and 35 are present in the N-, B- and NB-tropic viruses of BALB/c origin that have been analyzed. Curiously, oligonucleotides N and 12 were found to share 10 bases of their sequence, although these are not the same 10 bases shared by the three tropism oligonucleotides. In a random arrangement of 10⁴ bases, the probability of a 10 base repetition is 10⁻⁴. The possible significance of the sequence repetition found in the N and 12 oligonucleotides, which lie in opposite thirds of the genome, is not known.

It is possible to alter the tropism of an MuLV by forced passage through mouse cells of incompatible Fv-1 type. Of the four host range conversions that should be readily detectable in vitro (B→N, N→B, B→NB, N→NB), only one, the B→NB conversion, has been observed (J. W. Hartley and W. P. Rowe, personal communication). NB-tropic viruses can be detected at a frequency of approxi-

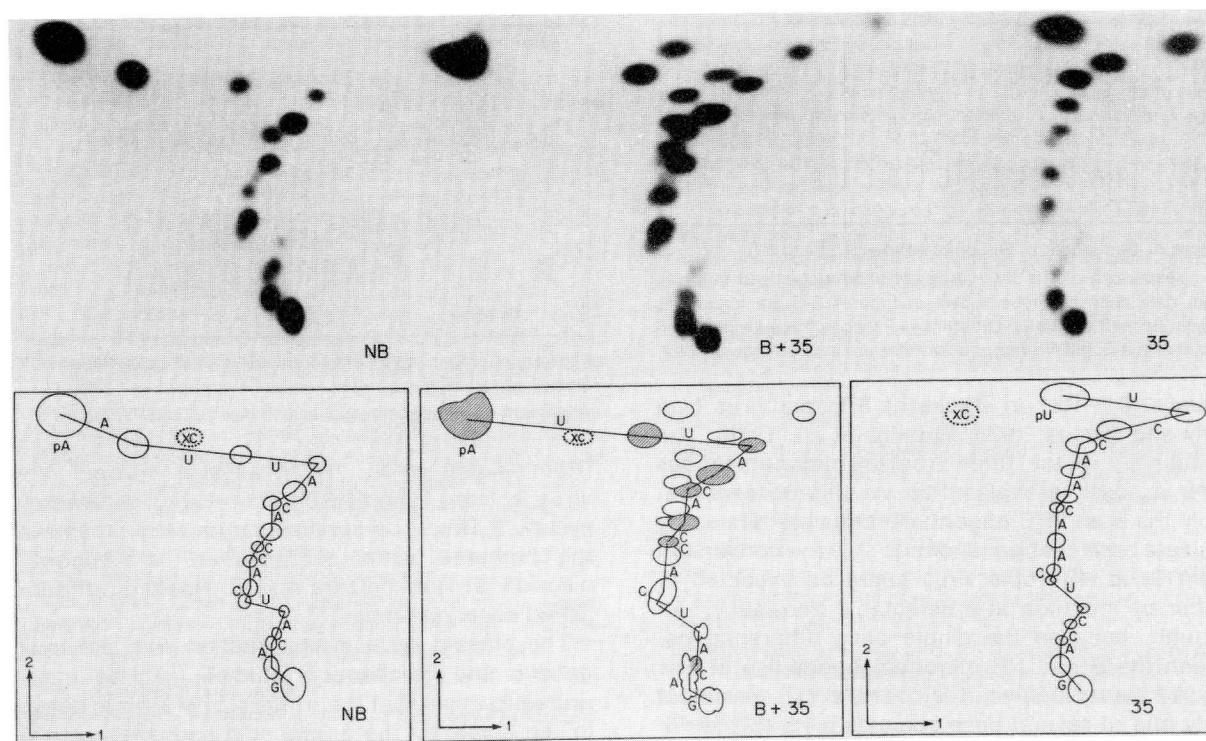


Figure 4. Sequence Analysis of Oligonucleotides 35, B+35 and NB

Autoradiograms and corresponding diagrams of the products of partial nuclease P1 digestion of 5'-³²P-end-labeled oligonucleotides after electrophoresis (arrow 1) and homochromatography (arrow 2). Shaded areas in the diagram of B+35 represent partial P1 digestion products not obtained from oligonucleotide 35 and therefore assumed to belong to the B oligonucleotide. (XC) indicates position of dye marker, xylene cyanol. Note the presence of a minor spot migrating at the position of adenylic acid in the digest of oligonucleotide 35. The origin of this "contaminant" is unknown.

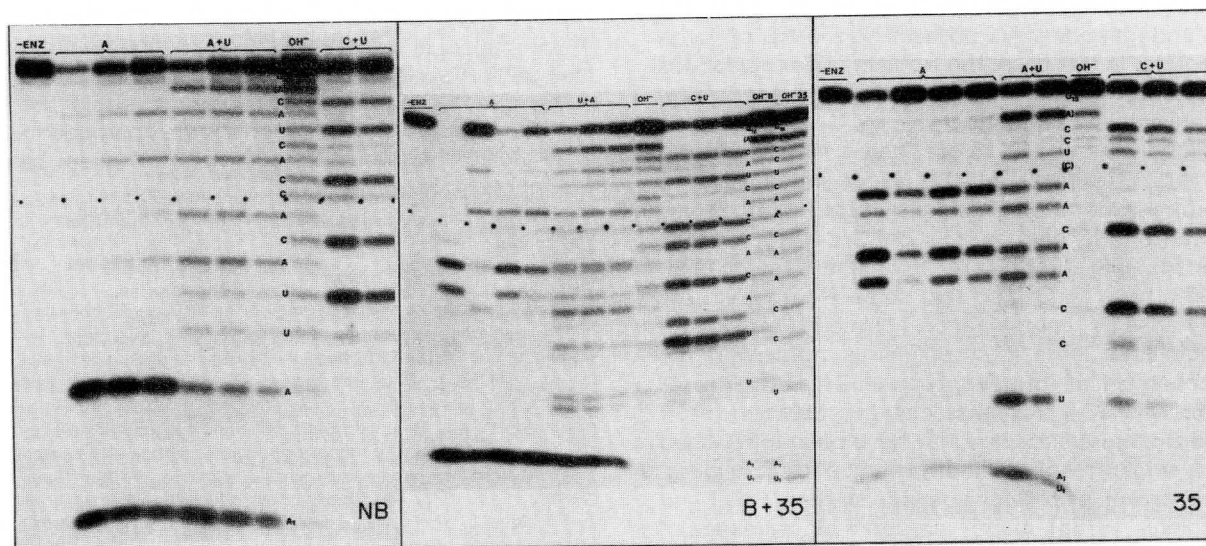


Figure 5. Sequence Analysis of Oligonucleotides 35, B+35 and NB

Autoradiograms of 5'-³²P-end-labeled T1 oligonucleotides after partial enzymatic cleavage or limited alkaline hydrolysis followed by electrophoresis. Cleavage specificity for (A), (A+U) and (C+U) as in Figure 3. (OH⁻ NB) products of limited alkaline hydrolysis of oligonucleotide 35, obtained from an NB-tropic viral RNA. (OH⁻ B) products of limited alkaline hydrolysis of the mixture of oligonucleotides B+35 obtained from B-tropic viral RNA.

There is a faint contaminating band in the NB oligonucleotide. Also note the presence in oligonucleotide 35 of a minor digestion product corresponding to adenylic acid. The origin of this contaminant is unknown.

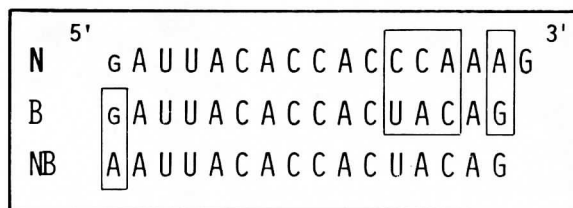


Figure 6. Sequences of Oligonucleotides N, B and NB
The sequences of the three tropism oligonucleotides deduced from data such as those shown in Figures 2-5 are aligned to maximize their similarity. Differences in sequence between the N and B or the B and NB oligonucleotides are indicated by boxes.

mately 10^{-5} – 10^{-6} in stocks of B-tropic virus (see Lilly and Pincus, 1973; Hopkins et al., 1976). The sequences of the three tropism oligonucleotides offer a possible explanation for this observation. Only the B and NB oligonucleotides are related by a single base change, and thus only the conversion from B- to NB-tropic virus might be expected to occur by mutation at a detectable frequency. (Although, because the single base difference between the B and NB oligonucleotides lies at one end of the molecules, it is possible that we detect only one of several base changes lying to their 5' side.) If NB-tropic viruses do arise from B-tropic viruses by a single point mutation, then the B and NB oligonucleotides must contain at least part of a nucleotide sequence that determines tropism. It should be noted that these data do not exclude a recombinational origin of NB-tropic viruses—for example, between an infecting B-tropic virus and an endogenous virus possessing the NB oligonucleotide. In this case, the tropism oligonucleotides might not reside in, but merely lie near to, sequences that determine tropism.

Robbins et al. (1977) obtained evidence that the B-tropic virus of BALB/c is not inherited as a chromosomally integrated provirus, but rather may arise from the endogenous BALB/c N-tropic virus. Benade, Ihle and Declève (1978) and Gautsch et al. (1978) have suggested that some B-tropic viruses may arise by recombination between N-tropic ecotropic virus and a xenotropic virus. If this were the mechanism of formation of the BALB/c B-tropic virus, then the four base changes between the B and N oligonucleotides may have been introduced in a single (recombination) event. This consideration, as well as previous genetic studies (Faller and Hopkins, 1978b), merely support a close linkage of the N and B oligonucleotides with a determinant of tropism, but do not help us to decide whether they lie within this determinant.

It is interesting to note that the N and B oligonucleotides are not unique to N- and B-tropic viruses of BALB/c. Akv, the N-tropic virus of AKR mice, possesses the N oligonucleotide as noted above

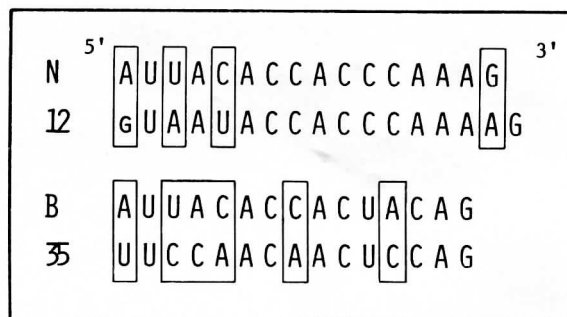


Figure 7. Comparison of the Sequences of Oligonucleotides N and 12, B and 35

Alignment of sequences and placement of boxes as in Figure 6.

(Rommelaere et al., 1977), while a B-tropic Akv virus isolated from AKR Fv-1^{bb} mice (J. W. Hartley and W. P. Rowe, personal communication) has lost the N oligonucleotide and acquired the B oligonucleotide (J. Rommelaere and N. Hopkins, unpublished observations).

The present data, in conjunction with previous genetic and biochemical studies, can be interpreted to mean that the tropism of MuLVs is determined solely by the amino acid sequence of p30 and that the three tropism oligonucleotides lie in or near p30 coding sequences (Hopkins et al., 1977; Schindler et al., 1977; Faller and Hopkins, 1977a, 1978b; Gautsch et al., 1978; J. Schindler, J. W. Gautsch and N. Hopkins, manuscript in preparation.) Confirmation of these possibilities awaits the demonstration of a role for p30 in the replication of MuLVs and its inhibition by an incompatible Fv-1 gene. It also awaits more precise localization on the genome of p30 coding sequences and of the tropism oligonucleotides, or possibly a comparison of amino acid sequences of p30 with the nucleotide sequences reported here.

Experimental Procedures

Viruses

The origins of the viruses from which the RNAase T1-resistant oligonucleotides were obtained for sequencing have been described previously as indicated: the N oligonucleotide was obtained from MCF v2 34 (Hartley et al., 1977), where it was designated oligonucleotide 39 (Rommelaere et al., 1978); oligonucleotide 12 was obtained from LP-B and JH-NB-clone 1 (Faller and Hopkins, 1977a); the mixture of oligonucleotides N+12 was obtained from Akv, where they were designated 39+39a (Rommelaere et al., 1977); the NB oligonucleotide was obtained from JH-NB-clone 1, where it was designated oligonucleotide NB-34; oligonucleotide 35 was obtained from JH-NB-clone 1 and Akv; and the mixture of oligonucleotides B+35 was obtained from LP-B virus, where the B oligonucleotide was designated B-35. Akv and MCF v2 34 viruses are derived from genetic information specified by the Akv loci of AKR mice (Chattopadhyay et al., 1975); the other viruses are derived from the BALB/c mouse.

Preparation of Viral RNA

70S viral RNA was prepared as described (Faller and Hopkins,

1977a), except that incubation with radioactive phosphate was omitted and the RNA was precipitated without carrier tRNA.

Isolation of 5' End Group-Labeled T1 Oligonucleotides

The preparation and 5' terminal labeling of RNAase T1-resistant oligonucleotides were essentially as described by Frisby (1977). Typically, 5 µg of viral RNA were digested with 5 units of RNAase T1 (Sankyo) in the presence of $5 \cdot 10^{-3}$ units of bacterial alkaline phosphatase (BAP.F; Worthington) for 30 min at 37°C in 10 µl of 25 mM Tris-HCl (pH 7.8), 1 mM $MgCl_2$. T1 oligonucleotides recovered after precipitation with 4 vol of ethanol were 5' end-labeled by incubation with 250 µCi of γ - ^{32}P -ATP (2500-3000 Ci/mM; New England Nuclear) and 2 units of polynucleotide kinase (P-L Biochemicals) for 20 min at 37°C in 50 µl of 50 mM Tris-HCl (pH 7.4), 0.01 mM EDTA, 0.5 mM spermidine, 10 mM $MgCl_2$, 5 mM dithiothreitol and 5% glycerol. The resultant ^{32}P -labeled RNAase T1 digest was fractionated by two-dimensional polyacrylamide gel electrophoresis as described (Faller and Hopkins, 1977a); the oligonucleotides were located by autoradiography and cut out using a 5 mm diameter cork borer. Virtually complete recovery of RNA from the gel was obtained using the crush-and-soak method (Maxam and Gilbert, 1977). The oligonucleotides typically possessed about 100,000 Cerenkov cpm. Some T1 oligonucleotides were contaminated, presumably by closely migrating oligonucleotides, and had to be further purified before sequencing by electrophoresis either on the same gel as used for the second dimension of the fingerprint but containing 6M urea, or on the sequencing gel for 9 hr at 1000 V (Donis-Keller et al., 1977).

RNA Sequence Determination

Two-Dimensional Fractionation of Partial Nuclease P1 Digestion Products (Silberklang et al., 1977)

The procedure was as described by Silberklang et al. (1977). Briefly, the 5'- ^{32}P -labeled T1 oligonucleotides and carrier tRNA (1 µg/µl) were incubated with nuclease P1 (Yamasa Shoyu) at a ratio of 2.5 µg enzyme per 10 µg RNA in 50 mM ammonium acetate buffer (pH 5.3) at 20°C. A good distribution of partial degradation products was obtained by pooling aliquots removed from the reaction at 6, 12 and 20 min. The RNA digests (at least 10,000 Cerenkov cpm, 3-6 µg RNA) were fractionated by electrophoresis on cellulose acetate strips (Schleicher and Schuell) at pH 3.5 in the first dimension, and by chromatography on 250 µm layers of cellulose MN 300 HR/MN 300 DEAE=15/2 (Analtech) in 50 mM KOH strength 3% homomix at pH 4.6 in the second dimension. The migration distances of the xylene cyanol dye spotted alongside the sample were 9.5 and 14 cm in the first and second dimensions, respectively.

Base-Specific Partial Enzymatic Cleavage and Fractionation of Products on Denaturing Polyacrylamide Gels (Donis-Keller et al., 1977)

5'- ^{32}P -end-labeled T1 oligonucleotides were sequenced directly using the method of Donis-Keller et al. (1977) with the following additions. Base-specific cleavage (U+A) was obtained from an enzyme mixture (Phy M) isolated from the culture broth of *Physarum polycephalum* (H. Donis-Keller, manuscript in preparation). Reaction conditions and buffer for partial enzymatic cleavage using Phy M were the same as those described by Donis-Keller et al. (1977) for RNAase U2. Aliquots of a *B. cereus* enzyme (Lockard et al., 1978) preparation (provided by J. Heckman) were incubated at 50°C for 15 min in a buffer containing 5'- ^{32}P -end-labeled oligonucleotide, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) and 5 µg carrier tRNA in a reaction volume of 10 µl. An equal volume of 10 M urea, 0.02% xylene cyanol, bromophenol blue was added to the *B. cereus* reaction mixture prior to loading onto the sequencing gel. Electrophoresis at 1000 V (constant power) was terminated after about 4.5 hr when the xylene cyanol dye reached 9.5 cm from the origin.

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