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A novel oncogene related to c-*mil* is transduced in chicken neuroretina cells induced to proliferate by infection with an avian lymphomatosis virus

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Non-dividing neuroretina cells from chicken embryos are induced to proliferate after a long latency, following infection with Rous associated virus type 1, an avian retrovirus which does not carry a transforming gene. We have isolated from these proliferating cells an acutely mitogenic retrovirus, designated IC10, which contains a novel oncogene. Nucleotide sequencing showed that the IC10 virus has transduced 1101 nucleotides of cellular origin inserted between the *gag* and *env* genes of RAV-1. This oncogene, designated v-R*mil*, is 70.1% homologous to v-*mil*. v-R*mil* encodes a protein of 40 976 daltons sharing 83.8% homology with the catalytic domain of the v-*mil* protein. Divergence with the v-*mil* gene product is observed at the NH_2- and COOH-terminal portions of the v-R*mil* protein. Restriction analysis of normal chicken DNA indicated that v-R*mil* is derived from a cellular gene distinct from c-*mil*. The c-R*mil* gene is transcribed through a major mRNA, >10 kb in length, that is detected at much higher levels in neuroretinas, as compared to other embryonic tissues.

Key words: cell division/neuroretina/oncogene/retrovirus/transduction

Introduction

Eukaryotic cells contain genes, named proto-oncogenes, which have the potential to transform cells *in vitro* and induce tumours *in vivo* (Bishop, 1983). These oncogenes were initially identified through their association with retroviruses which acted as natural transducing vectors (Stehelin *et al.*, 1976). It is now believed that oncogene products are components of signal transducing pathways and play a role in regulation of cell growth and differentiation (Bishop, 1985).

We have previously shown that several acutely transforming retroviruses induce proliferation of avian embryonic neuroretina (NR) cells which normally do not divide *in vivo* or *in vitro* and that cell multiplication requires expression of the oncogenes present in these retroviruses (Pessac and Calothy, 1974; Calothy *et al.*, 1980). Therefore, these differentiating cells constitute a useful host system to study the effects of oncogenes on cell growth regulation.

We report that the avian lymphomatosis virus (ALV), Rous-associated virus type 1 (RAV-1), which does not carry an oncogene, also induces sustained growth of chicken embryo NR cells. However, multiplication of NR cells infected with RAV-1 is observed after a long latency and presumably results from the activation of cellular genes in a minority of infected cells. We have isolated from proliferating NR cells, infected with RAV-1, an acutely mitogenic virus containing a novel oncogene and determined its nucleotide sequence. The transduced gene is a member of the *mil/raf* family of serine/threonine protein kinases and was, therefore, designated v-R*mil*. We also show that normal chicken DNA contains sequences homologous to v-R*mil* that are transcribed at higher levels in NR cells, as compared to other embryonic tissues.

Results

Isolation of an acutely mitogenic virus from NR cells infected with RAV-1

NR cultures dissected from 8-day-old chicken embryos are exclusively composed of differentiating neurones and glial cells that rapidly cease to divide and cannot be propagated *in vitro* (Crisanti-Combes *et al.*, 1977). Several cultures of NR cells were infected with a stock of RAV-1 purified by end-point dilution in chick embryo fibroblasts (CEF). As previously reported (Calothy and Pessac, 1976), RAV-1-infected NR cells were initially indistinguishable from uninfected cells in their normal morphology and limited growth capacity. However, after 4–6 weeks, one or two areas of multiplying cells were observed in about 2/3 of infected dishes. They initially consisted of a small number of flat cells with an epithelial-like morphology. In most dishes, NR cells ceased to divide after one passage, whereas in a few others cell proliferation continued for 10–15 generations.

Medium harvested from one culture which actively proliferated was used to infect fresh NR cells. This time, foci of multiplying cells were observed in all infected dishes after 3–4 weeks. Infected cultures were pooled and virus collected from these multiplying cells was used to infect fresh NR cells. This procedure was repeated four additional times. Culture fluids collected after the fifth passage of supernatants on NR cells were able to induce cell multiplication within one week, suggesting that they contained an acutely mitogenic virus(es).

Molecular cloning of IC10 provirus

To identify the mitogenic component(s), DNA extracted from NR cells induced to multiply at each virus passage, was digested with restriction enzyme *Eco*RI and analysed by Southern blot hybridization, using an LTR probe (Figure 1A). All infected NR cells contained the 2.4 and 1.3 kb DNA fragments corresponding to the 5′ and 3′ portions of the RAV-1 genome, respectively. However, *Eco*RI-digestion of DNAs from NR cells infected at the fifth and sixth passages of supernatants generated an additional fragment of 4.2 kb

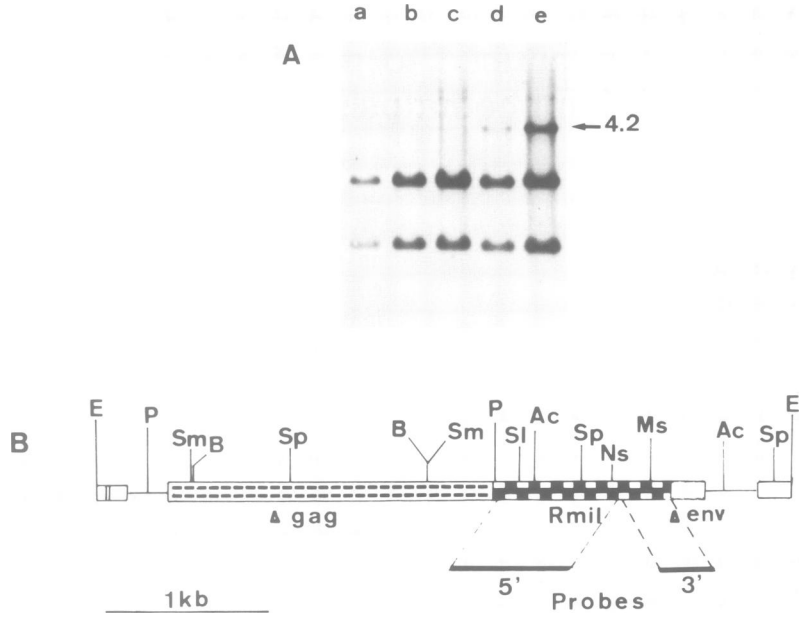

Fig. 1. Genetic structure of IC10 provirus. (**A**) Southern blot analysis of DNAs from RAV-1-infected NR cells. DNA was extracted from NR cells infected at the second (a), third (b), fourth (c), fifth (d) and sixth (e) passages of virus on fresh NR cultures. Aliquots (10 μg/lane) were digested with *Eco*RI, electrophoresed on 1% agarose gels, blotted onto nitrocellulose filters and hybridized to ^{32}P-labelled LTR probe, under stringent conditions. The size of DNA fragments is expressed in kilobases. (**B**) Restriction map of a molecular clone of IC10 provirus. DNA of λC125, a biologically active molecular clone of IC10, was digested with different restriction enzymes, electrophoresed on 1% agarose gels, blotted and hybridized to *gag, pol, env* and LTR radioactive probes. Restriction enzymes used were: *Eco*RI (E), *Sph*I (Sp), *Pst*I (P), *Bam*HI (B), *Msp*I (Ms), *Sal*I (Sl), *Nsi*I (Ns), *Acc*I (Ac) and *Sma*I (Sm). v-R*mil* specific probes were prepared as follows: the 5′ probe was obtained by subcloning the *Pst*I–*Nsi*I DNA fragment of 730 bp into the unique *Pst*I site of Bluescript plasmid; the 3′ probe was obtained by subcloning the *Nsi*I–*Acc*I fragment of 640 bp into the *Sma*I site of Bluescript plasmid and subsequent digestion of the DNA with exonuclease III.

which also hybridized to a *gag*-specific probe (data not shown). This DNA fragment was not detected in NR cells infected at earlier passages of the virus.

A genomic library was obtained from NR cells infected at the sixth passage of virus-containing supernatants. High-molecular-weight DNA was partially digested with *Eco*RI, ligated to purified arms of λgt11 DNA and packaged *in vitro*. Recombinant phages containing viral sequences hybridizing to LTR and *gag* probes were selected, further purified and amplified. One clone, λC125, harboured the 4.2 kb *Eco*RI fragment. NR cells transfected with both λC125 and helper RAV-1 DNA were induced to proliferate and generated the 4.2 kb fragment upon digestion of cell DNA with *Eco*RI. In addition, culture fluids from these transfected cells contained virus that rapidly induced NR cell multiplication (data not shown). This cloned mitogenic virus was designated Institut Curie 10 (IC10).

The genetic structure of the IC10 provirus was determined by Southern blot analysis of λC125 DNA digested with various restriction enzymes and hybridized to LTR, *gag*, *pol* and *env* specific probes (Figure 1B). A *Pst*I site approximately located in the middle of the IC10 genome divides the provirus in two portions. A 5′ portion of 2.4 kb containing LTR and *gag* sequences and a 3′ portion of 1.9 kb hybridizing only to the LTR probe. These data suggested that the 3′ portion of IC10 provirus contains additional sequences presumably responsible for the mitogenic property of this virus. In addition, these sequences did not hybridize to probes specific for the following oncogenes: v-*src*, v-*mil*, v-*erb*B and v-*myc* (data not shown).

Nucleotide sequence analysis of cell-derived sequences of IC10 provirus

The 4.2 kb insert of λC125 was subcloned into the unique *Eco*RI site of Bluescript plasmid and the entire sequence of IC10 provirus was determined on both strands by the method of Sanger (1981). The nucleotide sequence of a portion of this provirus, comprised between the second *Bam*HI site of the *gag* gene and the *Acc*I site at the 3′ end of the provirus is presented in Figure 2.

A single long open reading frame (ORF) extends from nucleotide −400 to nucleotide +1302. It includes 133 amino acids of *gag* fused with 367 amino acids of cellular origin. The last 67 amino acids and the stop codon are provided by RAV-1 *env* sequences. The cell-derived sequences extend from nucleotide +1 to nucleotide +1101 and show a 70.1% homology with the v-*mil* oncogene of MH2 (Galibert *et al.*, 1984; Kan *et al.*, 1984). We have, therefore, named this new oncogene v-R*mil*. The caculated molecular mass of the 367-amino acid protein encoded by v-R*mil* is 40 976 daltons.

Comparison of the deduced v-R*mil* amino acid sequence to that of the v-*mil* protein of MH2 showed an overall homology of 69.9% (Figure 3). A homology of 83.8% is observed between amino acids 72 and 343, corresponding to the catalytic domain of v-*mil*. This region contains the putative ATP-binding domain conserved in the *raf/mil* gene family (Beck *et al.*, 1987), including a lysine at position 93 involved in ATP-binding that is also found in members of the tyrosine kinase gene family (Kamps *et al.*, 1984). At the NH_2-terminal portion of the v-R*mil* protein (amino acids 1–71), homology with the v-*mil* product is only 28.2%,

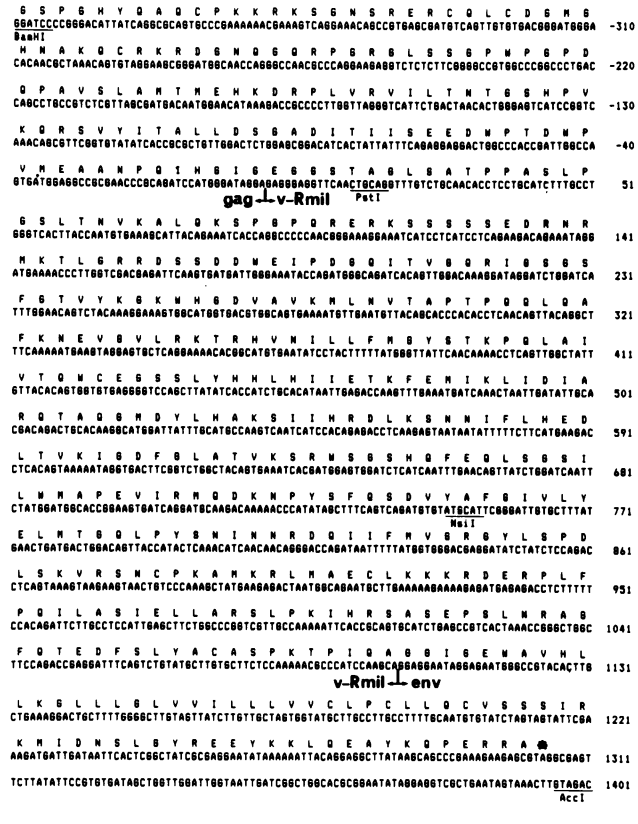

Fig. 2. Nucleotide sequence of the *Bam*HI−*Acc*I portion of IC10 provirus containing the v-R*mil* oncogene. The sequence was determined on both strands by the dideoxynucleotide-chain termination method following subcloning into the Bluescript vector and exonuclease III deletions. The predicted amino acid sequence of the unique ORF is shown above using single letter amino acid notation. The stop codon of this ORF is indicated by an asterisk. Numbers at the end of each line refer to nucleotides with position +1 defining the beginning of the v-R*mil* gene. Junctions between *gag*, v-R*mil* and *env* genes are indicated. The 5′ recombination site between *gag* and v-R*mil* sequences was deduced by comparison with the *gag* sequence of PrC strain of Rous sarcoma virus. The 3′ recombination site between v-R*mil* and *env* sequences was deduced by comparison with the *env* sequence of RAV-2.

Fig. 3. Amino acid sequence homology between the v-R*mil* gene and the v-*mil* gene of retrovirus MH2. Dashed lines indicate gaps introduced to align sequences. Open spaces represent amino acid identities with respect to v-R*mil*. The sequence representing the putative ATP-binding domain is marked by the broken line on the top of the R-*mil* sequence. The conserved lysine of this domain (corresponding to Lys 622 of the v-*mil* product) is indicated by an arrow and the stop-codon of v-*mil* by an asterisk.

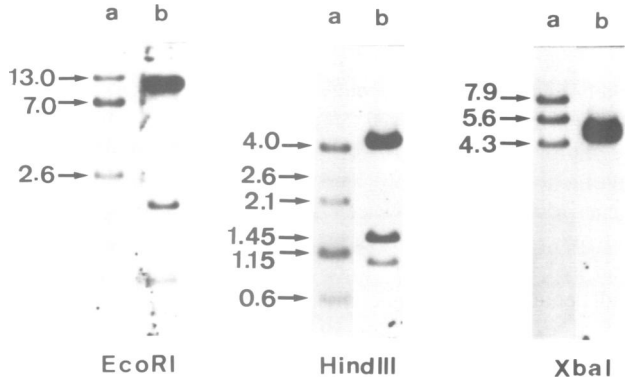

Fig. 4. Identification of c-R*mil* sequences in normal chicken cells. DNA (10 μg/lane) extracted from 11-day-old chicken embryos was digested with restriction enzymes *Eco*RI, *Hin*dIII and *Xba*I, fractionated on 1% agarose gels, blotted and hybridized to ^{32}P-labelled 5′ and 3′ v-R*mil* probes (**lane a**) and to a v-*mil* specific probe (**lane b**). Derivation of the 5′ and 3′ v-R*mil* probes is described in the legend of Figure 1. The size of DNA fragments is expressed in kilobases.

whereas at the COOH-terminus (amino acids 344−367) it is 31.8%.

Identification of c-Rmil sequences in normal chicken cells

Genomic DNA, extracted from 11-day-old chicken embryos, was analysed by Southern blotting following digestion with restriction enzymes *Eco*RI, *Hin*dIII and *Xba*I. Blots were hybridized to ^{32}P-labelled 5′ and 3′ v-R*mil* probes and to ^{32}P-labelled v-*mil* probe. The v-R*mil* 5′ and 3′ probes detected *Eco*RI fragments of 13.0, 7.0 and 2.6 kb; *Hin*dIII fragments of 4.0, 2.6, 2.1, 1.45, 1.15 and 0.6 kb and *Xba*I fragments of 7.9, 5.6 and 4.3 kb. These DNA fragments are different from those of the chicken c-*mil* DNA, generated by the same restriction enzymes (Figure 4). Therefore, normal chicken DNA contains 12−22 kb of sequences, designated c-R*mil*, homologous to v-R*mil*.

We also searched for c-R*mil* expression in different tissues from 11-day-old chicken embryos and in cultured CEF. Northern blots of polyadenylated RNAs, isolated from dissected NR, liver, lung, kidney and heart and from CEF were hybridized with the 5′ v-R*mil* probe. A major transcript, >10 kb in length, was detected in NR cells. This mRNA was also found in CEF and in the other tissues, but at considerably lower levels (Figure 5).

Discussion

We have shown that non-dividing NR cells from 8-day-old chick embryos acquire sustained growth capacity upon infection with RAV-1, an ALV which does not carry an oncogene. Furthermore, by serially passaging the supernatants from one proliferating culture onto fresh NR cells, we have isolated an acutely mitogenic virus, IC10, containing a novel oncogene related to c-*mil*.

In contrast to the rapid and massive proliferation resulting from the expression of retroviral oncogenes, multiplication of RAV-1 infected NR cells is observed after a long latency and initially occurs in a very small number of cells. It is

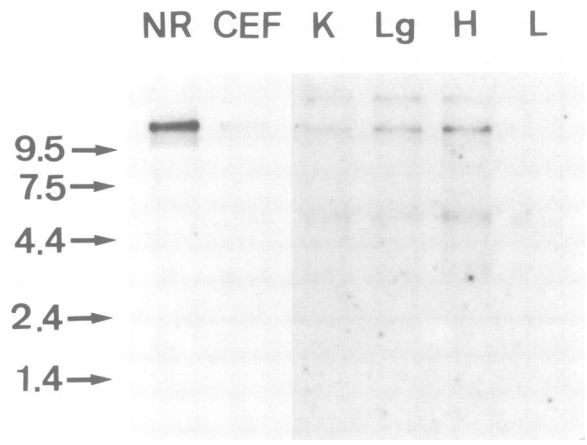

Fig. 5. Transcription of c-R*mil* sequences in chicken tissues. Polyadenylated RNAs were isolated from neuroretinas (NR), liver (L), heart (H), kidney (K), lungs (Lg) and cultured fibroblasts (CEF) of 11-day-old chicken embryos. RNAs (1 μg/lane) were denatured by formamide−formaldehyde treatment, fractionated on 1% agarose−2.2 M formaldehyde gels, blotted and hybridized with ^{32}P-labelled 5′ v-R*mil* probe; RNA ladder (Bethesda Research Laboratories) was run as size marker. The size of RNAs is expressed in kilobases.

likely that proliferation results from thus far undefined interactions between the RAV-1 genome and cell growth regulatory elements in a sub-population of NR cells. These interactions presumably lead to the activation of cellular genes responsible for NR cell division and to their subsequent retroviral transduction. Induction of NR cell proliferation and isolation of acutely mitogenic viruses was repeated in independent experiments (G.Calothy and D.Laugier, unpublished results). Interestingly, we have shown that in one such experiment,infection of NR cells with RAV-1 resulted in transcriptional activation and transduction of c-*mil*-derived sequences (Marx *et al.*, 1988). It thus appears that proliferation of NR cells infected with RAV-1 correlates with the activation of two related cellular genes. This model should prove useful in studying early steps of interactions between the RAV-1 genome and regulation of cellular gene expression.

The cellular sequences transduced in IC10 virus share a 70.1% homology with v-*mil*, which corresponds to the COOH-terminal portion of the c-*mil*/c-*raf* gene (Jansen and Bister, 1985; Bonner *et al.*, 1986; Koenen *et al.*, 1988) and a 65.5% homology with the COOH-terminal portion of the human A*raf* gene (Beck *et al.*, 1987) (data not shown). Therefore, v-R*mil* is a new member of this gene family encoding serine/threonine protein kinases. After completion of this manuscript, Ikawa *et al.* (1988) reported the identification of a third member of the *raf* gene family, designated B-*raf*. A comparison of the deduced amino acid sequence of the two genes showed a perfect homology between the corresponding portions of the two proteins (data not shown). Therefore, it is likely that v-R*mil* and B-*raf* are derived from two cognate genes in the chicken and human species. In addition, v-R*mil* is functionally related to v-*mil*, also shown to express mitogenic capacity in NR cells (Béchade *et al.*, 1985).

Restriction analysis of normal chicken DNA confirmed that v-R*mil* is derived from a cellular gene distinct from c-*mil*. The c-R*mil* gene is transcribed into a large mRNA, >10 kb in length. Hence, the transduced portion of c-R*mil*, which corresponds to ~1/10 of the spliced gene, is sufficient for the mitogenic property of the IC10 virus. This is in agreement with previous data showing that LTR-activation of the COOH-terminal portion of c-*mil* results in NR cell proliferation (Dozier *et al.*, 1987). By analogy with other members of the serine/threonine kinase oncogene family (Beck *et al.*, 1987), it is likely that the transduced portion of c-R*mil* also represents the COOH-terminal portion of this gene. Therefore, truncation of the c-R*mil* product could represent the mechanism of activation of the mitogenic property of this gene, suggesting that the non-transduced portion of the c-R*mil* product could regulate the biological properties of this protein.

The c-R*mil* transcripts differ markedly by their size from those of other members of the *mil/raf* family (Coll *et al.*, 1983; Mölders *et al.*, 1985; Ishikawa *et al.*, 1987). Therefore, we anticipate finding major structural differences between c-R*mil* and the other related genes. That the c-R*mil* gene is transcribed at higher levels in NR from 11-day-old embryos, as compared to CEF and other embryonic tissues suggests a role for this gene in regulating some essential function(s) in NR cells.

Materials and methods

Cell cultures and viruses

NR cultures were prepared from 8-day-old Brown Leghorn chick embryos (gs^+ chf^+) of the Edinburgh strain, as previously described (Pessac and Calothy, 1974). Cultures were maintained and passaged in Eagle basal medium supplemented with 5−8% fetal calf serum. CEF were prepared from 11-day-old embryos by standard procedures and grown in Dulbecco's modified Eagle medium containing 5% newborn calf serum, 1% heat-inactivated chicken serum and 10% tryptose phosphate broth. Rous-associated virus type 1 (RAV-1) is a subgroup A lymphomatosis virus routinely grown in CEF. Virus cloned by two cycles of end-point dilution on CEF was used as a source of RAV-1 in these studies. NR cells seeded in 35-mm dishes (2×10^6 cells per dish) were infected at a m.o.i. of ~0.1, as previously described (Pessac and Calothy, 1974).

DNA purification and restriction enzyme analysis

High-molecular-weight DNA was purified from cells by standard procedures (Gross-Bellard *et al.*, 1973). DNA of λ phages grown in *Escherichia coli* Y1090 was prepared as described (Maniatis *et al.*, 1982). Plasmid DNAs were purified according to the cleared-lysate method and centrifugation in CsCl−ethidium bromide gradients. DNAs were digested to completion with restriction endonucleases under conditions recommended by the suppliers (New England Biolabs and Bethesda Research Laboratories), fractionated by electrophoresis in 1% agarose gels and transferred to nitrocellulose filters in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) by the method of Southern (1975). Hybridization was performed under stringent conditions described by Wahl *et al.* (1979), using probes radioactively labelled by nick-translation (Rigby *et al.*, 1977).

RNA isolation and Northern blot analysis

Total cellular RNA was isolated by using the guanidium-thiocyanate-caesium chloride method (Chirgwin *et al.*, 1979). Polyadenylated RNAs were selected by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). The RNAs were denatured at 60°C in a formamide−formaldehyde mixture (Maniatis *et al.*, 1982), fractionated by electrophoresis in 1% agarose−2.2 M formaldehyde gels (Lehrach *et al.*, 1977), transferred to nitrocellulose filters in 20 × SSC (Thomas, 1980) and hybridized to ^{32}P-labelled probes.

Molecular probes

The following probes were used: (i) the 350 bp *Eco*RI fragment (EcoD) of long terminal repeat (LTR) sequences from the Rous sarcoma virus molecular clone pSRA2 (DeLozbe *et al.*, 1980; (ii) the 2.1 kb *Sac*I−*Eco*RI *gag*-specific fragment of plasmid pB5 *gag* (Stavnezer *et al.*, 1981); (iii) the 1.8 kb *Kpn*I−*Sac*I *env*-specific fragment of plasmid pB5 *env* (Stavnezer *et al.*, 1981); (iv) a plasmid containing *pol* sequences obtained by subcloning the 2.2 kb *Hin*dIII−*Kpn*I *pol*-specific fragment of plasmid pSRA2 into

pUC18; (v) the 1.1 kb *Bam*HI–*Hpa*I v-*mil* specific fragment of the pMH2BS molecular clone (Coll *et al.*, 1983).

Molecular cloning

High-molecular-weight DNA was partially digested with *Eco*RI and fractionated by centrifugation through sucrose gradients (Maniatis *et al.*, 1982). The 4–8 kb *Eco*RI DNA fragments were ligated to *Eco*RI digested arms of λgt11 (Young and Davis, 1983) and packaged *in vitro*, using a 'Gigapack plus' extract (Stratagene). Recombinant phages were selected by plaque-hybridization (Benton and Davis, 1977) with ^{32}P-labelled LTR and *gag* probes and further purified and amplified.

DNA sequencing

The selected recombinant phage DNA was digested to completion with *Eco*RI. The proviral insert was subcloned into the unique *Eco*RI of Bluescript plasmid (Stratagene). The DNAs of selected clones were digested with Exonuclease III to generate ordered deletions (Henikoff, 1984). The nucleotide sequence was determined on both strands by the dideoxy-chain termination method of Sanger (1981).

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