Restriction Enzyme Analysis of Partially Transformation-Defective Mutants of Acute Leukemia Virus MC29

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Restriction enzyme mapping and limited sequence analysis have been used to study the generation and genome structure of three partial-transformation mutants of avian acute leukemia virus MC29. The three mutants, *td10A*, *td10C*, and *td10H*, could be shown to have sustained overlapping deletions of 200, 400, and 600 base pairs, respectively, in their genomes. The precise location of the deletions was mapped within the *v-myc* gene of the mutants by limited sequence analysis of cloned MC29 DNA. The data obtained are discussed in terms of the effect of these deletions on the mechanism of transformation by MC29.

Avian myelocytomatosis virus MC29 is a replication-defective acute leukemia virus which induces a broad spectrum of diseases, including renal and liver tumors, myelocytomatosis, and, less frequently, sarcomas and erythroblastosis (1, 16). In vitro, MC29 induces the transformation of fibroblasts, as well as macrophages, from the bone marrow and yolk sac (11). Definition of the genome structure and analysis of proteins encoded by the virus showed that it has partial deletions in both *gag* and *env* genes. These partially deleted genes flank specific sequences, termed *myc* (2, 15, 20), which are represented in the only detectable protein encoded by this virus, a phosphorylated polyprotein of 110,000 daltons (p110) made up of *gag*-related peptides and *myc* peptides (3, 4, 12).

Recently, p110 was implicated as the transforming protein of MC29. For this we isolated a series of nonconditional mutants from an MC29-transformed quail fibroblast line, Q10. These mutants, although able to transform fibroblasts in vitro, did not transform macrophages from infected bone marrow (18). In vivo, they caused none of the tumors normally associated with wild-type MC29 (P. J. Enrietto, G. M. Ramsay, J. A. Wyke, M. J. Hayman, and L. N. Payne, submitted for publication). These transformation-defective mutants encoded smaller *gag*-related polyproteins of 100, 95, and 90 kilodaltons (kd) and could be shown to lack *myc*-specific peptides (19). In addition, the analysis of mutant viral RNAs by T1 oligonucleotide fingerprinting showed that the mutant viruses lacked *myc*-specific oligonucleotides (5).

The purpose of the studies described here was twofold. First, we were interested in determining whether the integrated proviruses of these mutants bore deletions, as the analyses up to

this point were restricted to protein and RNA only. Second, it was of interest to map the deletions within the *myc* gene as precisely as possible to identify the regions of the gene involved in macrophage and fibroblast transformation.

To this end, the wild-type MC29-transformed quail cell line Q8, as well as the mutant-infected quail fibroblast lines *td10A*, *td10C*, and *td10H*, were analyzed by the restriction enzyme mapping of isolated high-molecular-weight DNA. In addition, limited sequence analysis of a cloned wild-type MC29 provirus (22) was carried out to locate T1 oligonucleotides identified as missing from the mutants in earlier studies (5).

Quail fibroblast lines infected with wild-type and mutant MC29 viruses, as well as a normal quail cell line (QT35), have been described previously (3, 17). Cells were washed three times in phosphate-buffered saline, drained, and incubated in 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl containing 10 mM EDTA, 1% sodium dodecyl sulfate, and proteinase K (Boehringer Mannheim Corp.) to a final concentration of 100 µg/ml. Incubation was usually carried out overnight. DNA was then extracted with phenol once, phenol-chloroform-isoamyl alcohol (1:24:1) twice, and chloroform-isoamyl alcohol (24:1) once, ethanol precipitated, and spooled. After being washed in 70% ethanol, DNA was dissolved in 10 mM Tris-hydrochloride (pH 7.8)–10 mM EDTA. A 10- to 15-µg amount of DNA was digested with an appropriate amount of enzyme in a standard buffer of 6 mM Tris, 6 mM β-mercaptoethanol, and 6 mM MgCl₂, which was adjusted to the salt conditions recommended by the manufacturer with 4 M NaCl. Reaction volumes were 50 µl, and incubation was carried out at 37°C. Enzymes *EcoRI*, *PstI*, *KpnI*,

*Bam*HI, and *Cla*I were purchased from Boehringer Mannheim Corp. After digestion, the DNA was electrophoresed on 0.8% agarose gels or 1.0% agarose gels in a buffer made up of a 40 mM Tris base, 5 mM sodium acetate, and 1 mM EDTA. It was then transferred to nitrocellulose filters by the procedure of Southern (21). Filters were prehybridized and hybridized by a previously described procedure (6, 7). MC29 DNA was obtained in the form of pBR322 containing the complete MC29 genome which was subcloned from the chimeric phage λ MC38, previously described (22) and kindly provided by B. Vennström. A 1.5-kb *Pst*I fragment derived from MC38 and subcloned into pBR322 (*pmyc-Pst*) was also supplied by B. Vennström and was used as a probe for viral *myc* (23). p1320 *gag* was kindly provided by D. Chiswell and is made up of a 1,320-base-pair (bp) *Bam*HI fragment from the *gag* region of the Schmidt-Ruppin strain of Rous sarcoma virus cloned into pAT153 (7). Probes were nick translated with the Amersham nick-translation kit (Amersham, United Kingdom). The subcloned *Pst*I fragment from MC38 was sequenced on both strands from both the *Cla*I and the *Sal*I sites by the method of Maxam and Gilbert (14).

High-molecular-weight DNA was prepared from the nonproducer wild-type MC29-infected quail fibroblast cell line Q8 and nonproducer mutant-infected cell lines 10A, 10C, and 10H. Control DNA used in these studies was from QT35, an uninfected quail cell line (17), and from QT35 infected with a transformation-defective mutant of Rous sarcoma virus, *tdB77*, which lacks *myc*-specific sequences but has *gag* and *env* sequences. These controls allowed the identification of endogenous *c-myc* fragments and of fragments from the *gag* and *env* region of the viruses studied.

The digestion of these DNAs with several restriction enzymes is shown in Fig. 1. In each case, the digested DNA was run on agarose gels, blotted onto nitrocellulose, prehybridized, and hybridized as described above. The probe used in each case was *pmyc-Pst*I, which recognizes the *myc*-specific sequences. Others have reported that the size of the genome of wild-type MC29 is about 5.5 kb (9, 22). In addition, Vennström et al. (22) have reported the presence of a *Kpn*I in the long terminal repeats (LTR) which flank the integrated proviral DNA in a cloned wild-type MC29. Therefore, to size the integrated viral genomes the DNA was digested with *Kpn*I, (Fig. 1A). In Q8, we observed a v-*myc* fragment of about 5.5 kb which comigrated with an endogenous, c-*myc* fragment of the same size (QT35). In QT35/*tdB77* on longer exposure, an analogous endogenous band of 5.5 kb can be seen. Therefore, the 5.5-kb fragment identified in Q8

FIG. 1. Restriction enzyme mapping of wild-type and mutant proviral DNA. High-molecular-weight DNA isolated as described in the text was cleaved with the indicated enzymes; fragments were separated on 0.8% agarose gels, transferred to nitrocellulose, and probed with nick-translated *pmyc-Pst*I as described in the text. In each panel the following DNAs were digested: (1) Q8, (2) QT35, (3) QT35/*tdB77*, (4) 10A, (5) 10C, (6) 10H. (A) A *Kpn*I digest, (B) a double digest of *Kpn*I and *Eco*RI, (C) a *Bam*HI digest, (D) a *Pst*I digest, and (E) a double digest using *Bam*HI and *Cla*I. Sizes indicated are in kilobases. Fragments resulting from a *Hind*III digest of lambda phage DNA were used as molecular weight markers.

probably represents the whole viral genome cut in the flanking LTRs. The digestion of 10A, 10C, and 10H with *Kpn*I yielded fragments of 5.3, 5.1, and 4.9 kb, respectively, in addition to the endogenous 5.5-kb fragment. Assuming that the mutants also have a *Kpn*I site within the LTR, this digest shows that the integrated proviruses of the mutants have deletions of 200 bp (10A), 40 bp (10C), and 600 bp (10H). These sizes correspond quite closely to those determined by Bister et al. (5) in their analysis of viral RNA. These data, therefore, confirm and extend the protein and RNA studies of these transformation-defective mutants and show that the integrated proviral DNA is deleted.

Further digestions were carried out to place more precisely the deletions within the viral genomes. *Eco*RI cuts once in the genome within the *env* gene (13, 22); therefore, to place the deletions with respect to this RI site, double digestions were carried out using *Kpn*I and *Eco*RI. An example is shown in Fig. 1B. When probed with *pmyc-Pst*I, Q8 yielded a fragment of 4.0 kb, as well as the 5.5-kb c-*myc* fragment which does not contain RI sites. Mutants *td10A*, *td10C*, and *td10H* yielded the 5.5-kb c-*myc* fragment, as well as fragments of 4.0, 3.8, and 3.6

kb, respectively. This implies that the RI site lies about 1.3 kb from the 3' *Kpn* site contained in the LTR of Q8 and all of the mutants and that the deletion lies to the 5' side of the RI site (Fig. 2).

The digestion of the wild-type and mutant DNAs was then carried out with the enzyme *Bam*HI. It had previously been shown by Lautenberger et al. (13) that the digestion of DNA from a related wild type-infected quail cell line, Q5, with *Bam*HI gave one *myc*-specific fragment of 3.1 kb. In agreement with these data, Q8 also gave a *myc*-specific fragment of 3.1 kb, whereas *td10A*, *td10C*, and *td10H* gave *myc*-specific fragments of 2.9, 2.7, and 2.5 kb, respectively (Fig. 1C). The position of the *Bam*HI sites within the viral genome was determined in double digests with RI or *Pst*I after sizing the resulting fragments and probing with MC38 or *pgag* 1,320 (data not shown). This digestion allowed the placement of the deletions within the 3.1-kb *Bam*HI fragment.

Digestion with *Pst*I was then carried out to determine whether the deletions could be located more precisely. As can be seen in Fig. 1D, Q8 gave one *myc*-specific fragment of 2.5 kb, *td10A* gave a fragment of 2.3 kb, *td10C* gave a fragment of 2.1 kb, and *td10H* gave a fragment of 1.9 kb; the additional bands in the *td10A* track are probably due to partial digestion. The position of the additional *Pst*I sites (Fig. 2) was determined in double digests of *Pst*I and *Eco*RI and by probing with MC38 and *pgag* 1,320 (data not shown). This allowed the assignment of the deletions to a region between the *Pst*I sites within the *myc* and *env* genes of the viruses (Fig. 2).

From the data presented, it can be seen that these mutants are very similar to each other and the wild type with respect to all enzymes used in these analyses except *Pst*I. The *Pst*I sites appear to vary within the *gag* region at the 5' part of the proviral genome. *td10C* and *td10H* have each

lost a *Pst*I site to the right of the *Bam*HI site in *gag*. Because the parental cell line Q10 is infected with helper virus as well as wild-type MC29, this variation may reflect the genetic variation resulting from recombination with helper virus used in the formation of infectious pseudotypes (8).

The digestions described so far located the deletions in *td10A*, *td10C*, and *td10H* to a *Pst*I fragment of 2.5 kb (Q8) and an overlapping *Bam*HI fragment of 3.1 kb (Q8). Both fragments span *myc*, the *Pst*I site being at the 5' end of the gene and the *Bam*HI site being near the 3' end of the gene but within *env* (Fig. 2). Sequencing data within the *myc* gene (described below) of the clone *pmyc-Pst*I indicated the presence of a *Clal* site within the gene which divided it into two regions. Therefore, it was of interest to determine whether we could place the deletions with respect to the *Clal* site. A double digest was done using *Bam*HI and *Clal*, and, as can be seen (Fig. 1E), Q8 yielded fragments of 1.8 and 1.2 kb. However, *td10A*, *td10C*, and *td10H* yielded only the characteristic *Bam*HI fragments, implying that the mutants lacked the *Clal* site found in wild-type MC29, Q8. It is not clear from these data whether the mutants have deleted the *Clal* site or have sustained point mutations eliminating the *Clal* site. However, this information pointed to the region around the *Clal* as being of importance for further analysis.

The presence in the Q8 cell line of a provirus which has a *Clal* site and the fact that the mutants lacked this implied that the deletions or mutations could be found in this area. To determine whether the T1 oligonucleotides found missing in the RNA of all of the mutants could be mapped to the region around the *Clal* site, we carried out DNA sequencing around the *Clal* site, using the procedures of Maxam and Gilbert. As can be seen in Fig. 3, the DNA sequences homologous to the two T1 oligonucleo-

FIG. 2. Restriction enzyme maps of wild-type (wt) and mutant proviral DNA. Proviral DNA of wild-type and mutant MC29 viruses (10A, 10C, and 10H) was analyzed by restriction enzyme mapping. A schematic of the viral genome is indicated. Restriction enzyme sites along the proviral genomes are indicated by arrows. Enzymes used were RI (*Eco*RI), K (*Kpn*), B (*Bam*HI), P (*Pst*I), and C (*Clal*). Deletions are indicated by the hatched boxes.

tides missing from all of the RNA of the mutants, no. 1 and no. 26, could be found within 114 and 79 bp to the right of the *Cla*I site, locating the deletions even more precisely, within the *Bam*HI and *Pst*I fragments.

This work attempts to characterize further three partially transformation-defective mutants of MC29, *td10A*, *td10C*, and *td10H*. We wanted to determine whether they bore deletions in the *myc* gene, as had been implied by the analysis of their protein products and viral RNA (5, 19). In addition, we wanted to map the mutations as precisely as possible to define the regions of the genome important in determining the oncogenic spectrum *in vivo* and *in vitro*. Our restriction mapping studies allowed us to determine that the mutants were, in fact, mutants lacking 200 bp (*td10A*), 400 bp (*td10C*), and 600 bp (*td10H*) of DNA. These sizes correlate quite well with a reduction in size of 10,000, 15,000, and 20,000 daltons in the respective *gag*-related polyproteins encoded by these viruses (18).

The deletions were mapped more precisely to a region within the *myc* gene around a *Cla*I site, present in Q8 but absent from the mutants. It was not clear whether the mutants sustained point mutations eliminating the *Cla*I site or whether the deletions actually spanned this site, but the limited sequence data around the *Cla*I site of the clone *pmyc-Pst*I implied that the site had been deleted. All of the mutants lack *myc*-specific oligonucleotides no. 1 and no. 26 (5), which we were able to locate 114 and 79 bp to the 3' side of the *Cla*I site. In addition, mutant *td10A* had gained an oligonucleotide, termed 1a, (5), which was a truncated version of oligonucleotide 1, implying that the deletion ended within this oligonucleotide. Since the deletion in *td10A* is 200 bp, and oligonucleotide 1 is only 114 bp to the 3' side of the *Cla*I site, these data also would

support the conclusion that the deletion spans this site. Because the *Cla*I site maps approximately 850 bp from the 3' end of the *myc* gene and because the largest deletion studied was 600 bp, it appears that all of the deletions are internal to *myc*, not spanning the *myc-env* junction.

Each deletion mutant encoded a protein of the predicted size (with respect to the nucleotide sequence deleted); therefore it appears that the deletions must be in phase, giving rise to polypeptides containing *gag* and *myc* sequences 5' and 3' to the deletion. Since the mutants were selected for their ability to transform fibroblasts, it is possible that in-phase deletions were subsequently selected, the implication being that information at the 3' end of the *myc* gene is required for fibroblast transformation. It is, of course, also possible that some or all of the 5' sequences present in the mutants are necessary for fibroblast transformation. Under different selection pressure, it may be possible to select mutants with different properties *in vitro*.

It would be of interest to select virus recovered from Q10 for macrophage-transforming ability in the hope of finding mutants which lack fibroblast-transforming ability. The analysis of the region around the *Cla*I site in such mutants may further define this important region of the genome since we know that the deletions to the right of this site decrease macrophage-transforming ability. In this regard, it is of interest that the *Cla*I site found in *v-myc* occurs at an analogous site in *c-myc*, the normal cellular homolog. However, the *Cla*I site in *c-myc* is found at the junction of the gene with an intervening sequence (23). Others have reported that intervening sequences within genes separate domains of the protein products of the gene (10). It is possible that the *Cla*I in *v-myc* resides on the border of such a domain (i.e., for fibroblast

FIG. 3. Nucleotide sequence of the *v-myc* gene, around the *Cla*I site. Sequencing of *pmyc-Pst*I was carried out around the *Cla*I site within the *v-myc* gene as described in the text. The oligonucleotides found missing in all of the virus mutants, nos. 1 and 26, are indicated within the sequence.

transformation versus macrophage transformation). The analysis of this region in more detail in these mutants and new isolates should allow a more careful correlation of regions of the *myc* gene with biological activity *in vitro* and *in vivo*.

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