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Intracisternal A-Particle Genes in *Mus musculus*: a Conserved Family of Retrovirus-Like Elements

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The structural organization of intracisternal A-particle genes has been studied, using isolates from a mouse gene library in λ phage Charon 4A. The predominant gene form among the isolates was 7.3 kilobases (kb) in length. R-loops between the 7-kb (35S) A-particle genomic ribonucleic acid and several of these genes were colinear, with no visible evidence of intervening deoxyribonucleic acid sequences. One recombinant was found with an A-particle gene that contained a 1.7-kb deletion. Using the deletion as a reference, the deoxyribonucleic acid and ribonucleic acid homology regions were localized with respect to one another and to the restriction map: the 5′ terminus of the ribonucleic acid was several hundred base pairs within the 5′ end of the deoxyribonucleic acid homology region. Restriction endonuclease fragments encompassing the 5′ and 3′ regions of one 7.3-kb gene were separately subcloned into pBR322. Heteroduplexes between the two subclones revealed an approximately 300-base pair segment of terminally redundant sequences. The cloned 3′ fragment hybridized with restriction fragments from the 5′ end of several other A-particle genes, demonstrating the presence of common (though not necessarily identical) terminally repeated sequences. A-particle genes varied in the occurrence of specific restriction sites at characteristic internal loci. However, heteroduplexes between several variant 7.3-kb genes showed continuous homology regions even when spread under stringent hybridization conditions. The relative abundance of restriction site variants was highly conserved in 12 laboratory strains of *Mus musculus*, in embryonic and adult tissues of a single inbred strain, and in the SC-1 cell line of feral mouse origin, but appeared to differ in a feral Japanese substrain, *Mus musculus molossinus*. Some evidence suggests that subsets of A-particle genes may have similar flanking sequences. The results are discussed in terms of the evolution of this multigene family.

The genome of *Mus musculus* contains multiple copies (500 to 1,000 per haploid equivalent) of deoxyribonucleic acid (DNA) sequences related to the high-molecular-weight ribonucleic acid (RNA) of intracisternal type A particles (28). These sequences appear to be distributed throughout the mouse chromosome complement. Recently, we identified a class of A-particle genetic elements consisting of approximately 7-kilobase (kb) units interspersed among variable flanking regions of mouse DNA (29). Six different 7-kb genes, isolated as recombinants in λ phage Charon 4A, showed individual variation in some internal restriction enzyme sites but no major substitutions or deletions as judged by blot hybridization or heteroduplex analysis.

In the present study we have shown that the individual A-particle genes exhibit certain structural features generally associated with integrated retroviruses, i.e., colinearity with the 35S A-particle genomic RNA and the presence of terminal repeated sequences. As part of the study, a number of additional genes were isolated (including one form that contains a major deletion), and it is now possible to distinguish several variant subgroups based on differences in restriction endonuclease sensitivities at specific internal loci. The relative abundance of these subgroups appears to be highly conserved in laboratory and feral strains of *M. musculus*, consistent with the evolution of A-particle genes as endogenous cellular elements.

MATERIALS AND METHODS

Mouse strains and cell lines. Males of the following laboratory strains (41) of *M. musculus* were obtained through the National Institutes of Health Small Animal Section: A/He, AKR/N, BALB/c, CBA/N, DBA/2, C3H/He, C57BL, C58/J, NZB/N, RIIIS/J, 129/J, and NIH Swiss. Myeloma MOPC-104E (a subcutaneous transplant line) and a 3T3 embryo fibroblast line were derived from tissues of BALB/c mice (1, 33). The SC-1 tissue culture line (American Type Culture Collection, CRL 1404) was derived from a feral specimen of *M. musculus* trapped in California

(21). Liver DNA from a feral Japanese subspecies, *Mus musculus molossinus*, was kindly provided by R. Callahan, National Institutes of Health. The N4 neuroblastoma line (2) originated from a tumor of an A/J mouse.

Preparations of nucleic acids. High-molecular-weight DNA was prepared as described (28) from livers and other mouse tissues and from cell lines mentioned above. Sonication-resistant spermatid nuclei were obtained from BALB/c testis by the procedure of Meistrich (34) except that the homogenization medium was modified to contain 10% sucrose, 100 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (Tris)·hydrochloride (pH 7.5), 5 mM disodium ethylenediaminetetraacetic acid (EDTA), 0.05% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride, and all subsequent solutions contained 1 mM EDTA. 35S A-particle RNA was isolated from N4 cells (36).

Recombinant DNA clones. A mouse gene library, generously provided by J. Seidman, National Institutes of Health, was prepared from DNA of 14-day BALB/c embryos according to Maniatis et al. (31): fragments averaging 15 to 16 kb in size were selected from a partial *Hae*III digest and inserted in λ phage Charon 4A (48) via *Eco*RI linkers. Plaques on *Escherichia coli* DP50 *supF* were screened (6) with A-particle probes (see below). DNA was prepared from CsCl-banded phage. Recombinants λMIA2, -14, -21, and -63 have been described (29). Two *Hin*dIII fragments representing the entire A-particle gene sequence in one recombinant, λMIA14 (29), were recovered individually from agarose gels by binding on glass microbeads (47), and each was cloned into the *Hin*dIII site of pBR322 (43). Recombinant plasmids pMIA4 and pMIA6 were grown in *E. coli* RRI.

Restriction enzyme digestion and agarose gel electrophoresis. Restriction endonuclease digestions were carried out in buffers recommended by the supplier (New England Biolabs). Wild-type λ phage DNA was added to nuclear DNA samples as an internal control for digestion and to provide molecular weight standards. Electrophoresis of digests in 0.8 to 1% agarose gels, staining with ethidium bromide, and transfer to nitrocellulose sheets have been previously described (16, 29, 40).

Labeled probes for hybridization. 35S A-particle RNA was randomly cleaved with alkali to an average length of 200 to 300 nucleotides and labeled with adenosine 5'-[γ-^{32}P]triphosphate (32) to a specific activity of 3×10^7 to 6×10^7 cpm/μg. DNA fragments were labeled by nick translation using adenosine 5'-[α-^{32}P]triphosphate (38) to specific activities of 3×10^7 to 10^7 cpm/μg. The A-particle gene fragments in pMIA4 (7 kb, including about 1 kb of flanking sequence) and in pMIA6 (1.1 kb) were used individually or were mixed in equimolar proportions to provide a probe representative of the entire gene. Filter hybridizations and autoradiography were carried out as previously described (29).

Heteroduplex and R-loop analyses. Recombinant phages were lysed with EDTA and alkali, and DNA heteroduplexes were prepared and mounted by the formamide technique as described by Davis et al. (17). The heteroduplex mixture (35°C) contained 50% formamide (Fluka, purissima grade), 60 mM NaCl, 10 mM trisodium EDTA, and 90 mM Tris·hydrochloride (pH 8.5). The standard spreading mixture contained 40% formamide, 50 μg of cytochrome *c* per ml, 10 mM EDTA, and 10 mM Tris·hydrochloride (pH 8.5); the standard hypophase was 10% formamide, 1 mM EDTA, and 10 mM Tris·hydrochloride (pH 8.5). Progressively more stringent conditions for heteroduplex pairing were obtained first by increasing the formamide concentration to 80% and 50% in the spreading mixture and hypophase, respectively, while keeping the EDTA and Tris·hydrochloride constant, and second by a 2.5-fold reduction in the EDTA-Tris·hydrochloride concentrations at the higher formamide levels.

R-loops (45) between 35S A-particle RNA and λ phage recombinants were formed at 47°C in mixtures containing 70% formamide, 150 mM NaCl, 5 mM disodium EDTA, and 25 mM sodium PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 7.2) and spread from 50% formamide over a 10% formamide hypophase (other components as in the standard mixtures above). Polyadenylic acid [poly(A)] tails of R-looped molecules were identified by reaction with polybromodeoxyuridylic acid-tailed simian virus 40 DNA (5). The molecules were spread from a mixture of 40% formamide, 10 mM disodium EDTA, and 100 mM sodium TES [*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfuric acid], pH 7.2, over a hypophase of 10% formamide, 1 mM EDTA, and 10 mM sodium TES. This reaction was kindly performed by Richard Kramer, National Cancer Institute.

RESULTS

A previous study (29) established a "consensus" restriction map for an approximately 7-kb A-particle gene based on blot hybridization patterns obtained after digestion of total nuclear DNA (BALB/c myeloma). Six individual 7-kb genes isolated from a mouse embryo gene library all showed variation in one or more of the consensus sites. We have since examined 18 other recombinants from the mouse gene library by restriction mapping or heteroduplex analysis or both, attempting to select those that might contain alternative forms of A-particle genes. Eleven of the new recombinants proved to have truncated genes abutting one λ arm or the other; one represented a deletion variant, and the remainder contained complete 7-kb genes. Four of the latter showed a new *Eco*RI cleavage pattern, whereas the other two fell within the range of restriction site variability previously observed.

The deletion variant, λMIA48, and one of the new *Eco*RI site variants, λMIA62, were then selected, together with two previously isolated 7-kb genes, λMIA14 and λMIA21, for extended structural analysis.

Major structural features of 7-kb genes. (i) Orientation and restriction maps. A partial restriction map of the 7-kb genes is shown in Fig 1. In each instance ^{32}P-labeled 35S A-particle RNA reacted strongly with restriction fragments

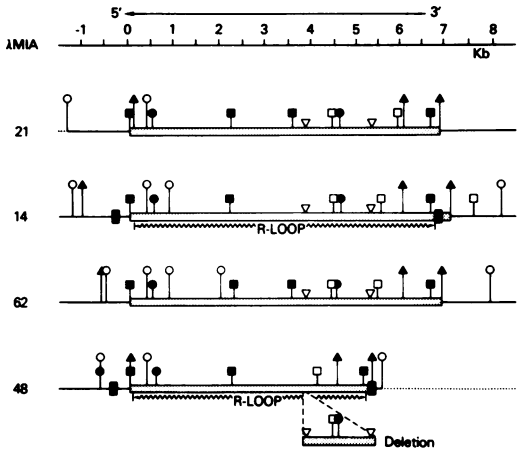

FIG. 1. *Physical maps of 10-kb regions containing the A-particle genes in four different λ Charon 4A recombinants, λMIA21, -14, -62, and -48. The extent of homology with 35S A-particle RNA as determined by restriction endonuclease cleavage and blot hybridization is represented by the stippled regions; solid lines indicate nonhomologous flanking regions of mouse DNA, and dotted lines indicate λ arms. The limits of the DNA homology region as mapped relative to the deletion in λMIA48 are indicated by heavy vertical bars (▮), and the extent of the RNA homology region as determined by R-loop analysis with 35S RNA is shown with wavy arrows (⇝). Restriction sites: ▲, HindIII; ○, EcoRI; ●, XbaI; ■, PstI; □, BglII; ▽, BamHI.*

lying between an invariant *Pst*I site on the left and a *Hin*dIII site 6.9 to 7 kb to the right. The 5′ ends of the genes (in terms of the RNA polarity) lie to the left. This orientation was established first by hybridization of restriction fragments with an oligodeoxythymidylic acid-primed complementary DNA probe enriched for 3′ sequences and confirmed by R-loop observation (see below). In view of these facts, the leftmost *Pst*I site has now been designated as map position 0, rather than one of the nearby *Eco*RI sites as in our previous study (29).

The mapping of the *Eco*RI sites has been clarified. λMIA21, and other genes which yield a 5.7-kb *Eco*RI/*Hin*dIII fragment, contain a single site at the 0.3-kb position. λMIA14 and other genes yielding a 5.2-kb *Eco*RI/*Hin*dIII fragment are now known to contain an additional *Eco*RI site at 0.8 kb, rather than a single site at a variable distance from the *Hin*dIII position as previously supposed (29). λMIA62 represents a new class of variant with a third internal *Eco*RI site at 1.9 kb. These genes yield a 4.1-kb *Eco*RI/*Hin*dIII fragment. Thus, 7-kb genes give rise to a series of progressively smaller *Eco*RI/*Hin*dIII fragments, all of which are prominent in blots of genomic DNA (see Fig. 5A).

A number of other constant or near-constant restriction sites have been seen. These include *Bam*HI sites at 3.8 and 5.2 kb, a *Bgl*II site at 4.3 kb, *Xba*I sites at 0.5 and 4.5 kb, a *Hin*dIII site at 6 kb, and a *Pst*I site at 6.6 kb. A *Pst*I site appears at 2.3 kb in the instances shown; however, genes lacking this site, as well as the one at 3.6 kb, have been observed. Of 15 complete or nearly complete genes now analyzed, 9 contained both internal *Pst*I sites, 3 lacked the site at 3.6 kb, 1 lacked the site at 2.3 kb, and in 2, both sites were absent.

(ii) Size of the DNA homology region. All 7-kb genes thus far examined, in this study and previously (29), regardless of internal variations in restriction sites, have formed continuous homology regions with one another on heteroduplex analysis, and none have shared flanking sequences as detected by this procedure. A heteroduplex between λMIA62 and λMIA2 (a previous isolate that yields a 5.2-kb *Eco*RI/*Hin*dIII fragment) is shown in Fig. 2A. Detailed measurements of the homology regions in this and three other pairings have now been made; they are λMIA14/λMIA21, 7.3 ± 0.2 kb; λMIA14/λMIA63, 7.3 ± 0.3 kb; λMIA21/λMIA63, 7.3 ± 0.4 kb; and λMIA62/λMIA2, 7.5 ± 0.3 kb. These values are consistently greater than the region of major homology as defined by blot hybridization with 35S A-particle RNA (6.9 to 7.0 kb).

Structure of a 5.6-kb A-particle gene. The recombinant λMIA48 showed a shortened homology region which lacked both internal *Bam*HI sites as well as the *Bgl*II and *Xba*I sites lying between them. The restriction data were consistent with a deletion as shown in Fig. 1, and this was confirmed by heteroduplex analysis (Fig. 3A). In this pairing, λMIA48/λMIA14, the measurements were: total homology region, 5.6 ± 0.2 kb; single-strand deletion loop, 1.7 ± 0.2 kb; and distance from deletion loop to the 3′ end of the DNA homology region, 1.4 ± 0.1 kb. The calculated length of the λMIA14 gene in this heteroduplex was 7.3 kb (5.6-kb homology plus 1.7-kb loop), showing that, aside from the deletion, the DNA homology region of λMIA48 was coextensive with that of the larger gene.

At present, there is no assurance that the shortened homology region contained in λMIA48 represents a true chromosomal gene rather than a cloning artifact. However, certain features of the genomic restriction patterns are consistent with the presence of deleted genes in the cellular DNA (see below).

Position of the A-particle DNA homology region. The known size and position of the deletion in λMIA48 made it possible to locate the DNA homology region with regard to the restriction maps of both this gene and λMIA14 (Fig. 1). We estimate from the heteroduplex data

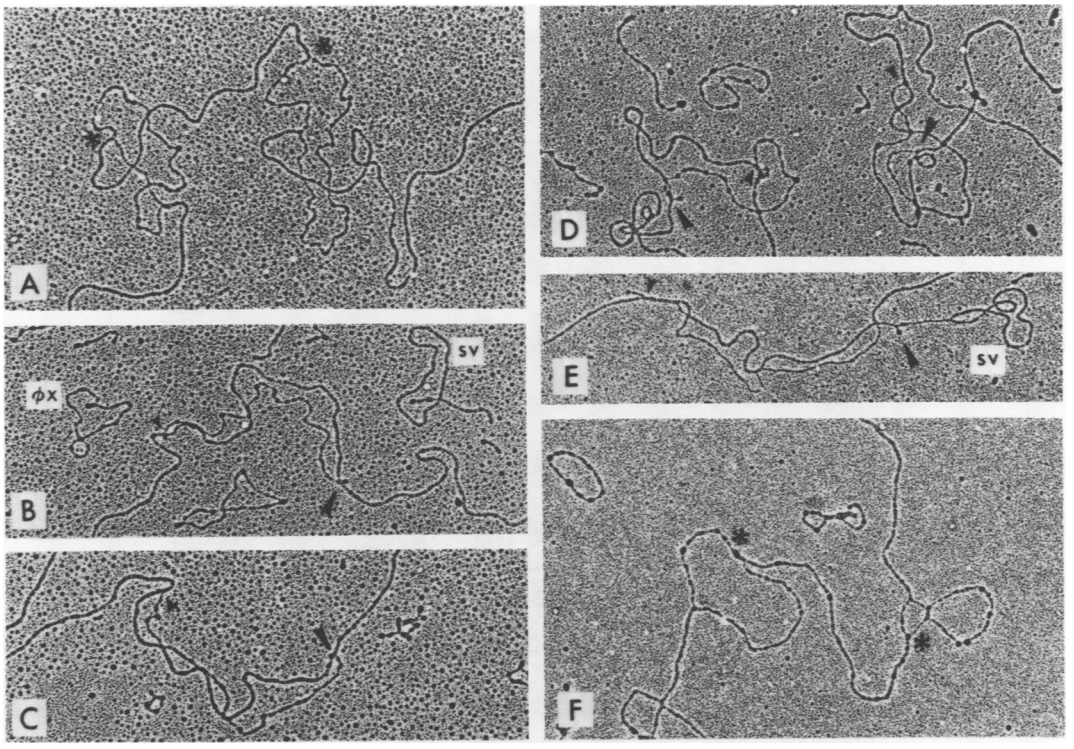

FIG. 2. *Representative heteroduplexes and R-loops formed with 7-kb A-particle genes. (A) A heteroduplex between λMIA62 (see Fig. 1) and λMIA2, a previously isolated recombinant (29) containing a gene similar to λMIA14 (but in the appropriate orientation to λMIA62). The junctions between the homology region and the nonhomologous flanking sequences are indicated (*). The heteroduplex was spread under standard conditions (see the text). (B) and (C) Two examples of R-loops formed between λMIA62 and 35S A-particle RNA. A putative poly(A) tail is seen in (B) but not in (C). 3' ends of the R-loop are indicated by large arrowheads; 5' ends are indicated by small arrowheads. Simian virus 40 and φX174 molecules, included as double- and single-stranded length standards (5,244 and 5,386 nucleotides, respectively), appear in (B) (SV and φX). (D) Two examples of R-loops between λMIA14 and 35S A-particle RNA. A putative poly(A) tail is seen in the left-hand example. (E) An R-loop between 35S RNA and λMIA62, in which the poly(A) tail has been identified unequivocally by reaction with a circular simian virus 40 molecule (SV) carrying a polybromodeoxyuridylic acid tail (see the text). Under the spreading conditions employed, there has been some displacement of the RNA molecule at either end of the R-loop. (F) A heteroduplex between λMIA14 and λMIA21, spread from 80% formamide (see the test).*

that the sequence homology begins approximately 0.4 kb to the left of the *Pst*I site at map position 0 and ends very near to the *Hin*dIII site at map position 7.0.

R-loop analysis. For sizing, 35S RNA from neuroblastoma A-particle was compared with avian myeloblastosis virus RNA by electrophoresis in agarose gels containing methyl mercury hydroxide (4). The A-particle RNA had the same mobility as the smaller avian myeloblastosis virus component, which is 7.0 kb in length (T. Papas, National Cancer Institute, personal communication). Allowing for a poly(A) tail of 0.2 kb (49), the transcribed portion of the 35S A-particle RNA was estimated at 6.8 kb.

R-loops were formed between this RNA and the DNAs of λMIA14, λMIA2, λMIA62, and λMIA48. Representative examples appear in Fig. 2B, C, and D and Fig. 3B and C. The three larger genes gave continuous DNA displacement loops with the following single-strand measurements: λMIA14, 6.6 ± 0.8 kb; λMIA2, 7.0 ± 0.5 kb; λMIA62, 6.6 ± 0.5 kb; average of all determinations, 6.7 kb. No intervening DNA sequences were observed. Poly(A) tails, mean size of 0.2 kb, were usually but not always visualized. They were unequivocally identified by reaction with polybromodeoxyuridylic acid-tailed simian virus 40 (Fig. 2E).

Typical R-loops between 35S RNA and the gene in λMIA48 are shown in Fig. 3B and C. The DNA displacement loop in these hybrids had a mean length of 5.1 ± 0.3 kb, the RNA loop corresponding to the deleted DNA region mea-

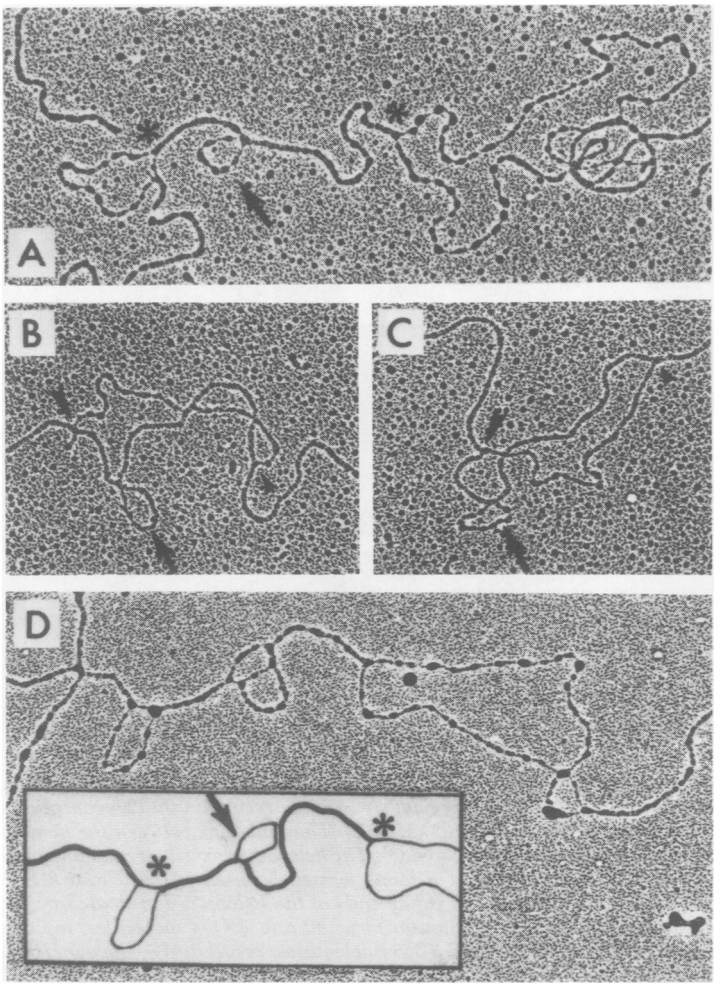

FIG. 3. *Heteroduplexes and R-loops formed with the 5.6-kb A-particle gene in λMIA48. This gene carries a 1.7-kb deletion (see Fig. 1). (A) Heteroduplex between λMIA48 and λMIA14, spread under standard conditions. Asterisks (*) mark the junctions between the gene homology region and the single-stranded loops formed by the nonhomologous flanking regions; the large loop on the right is partially tangled. A single-strand loop (arrow) is formed by λMIA14 DNA at the position of the 1.7-kb deletion in λMIA48. (B) and (C) R-loops formed between 35S A-particle RNA and λMIA48. A poly(A) tail is seen at the 3′ end in (B), but not in (C). In each case, the DNA:RNA hybrid strand shows a smaller single-strand loop (arrow) of RNA at the position of the deletion in λMIA48. (D) A heteroduplex between λMIA48 and λMIA14, but spread from 80% formamide (see the text). The location of the deletion loop is diagrammed in the figure insert.*

sured 1.6 ± 0.2 kb, and the distance from the deletion loop to the poly(A) tail along the hybrid strand was 1.3 ± 0.1 kb. The data are consistent with the dimensions obtained by heteroduplex analysis. The total RNA length, exclusive of the poly(A) tails, was 6.7 kb (5.1 plus 1.6), in agreement with the measurement obtained from R-loops with the larger genes.

Based on the known location of the deletion, the regions of homology visualized by R-looping were positioned on the restriction maps of λMIA48 and, by inference, λMIA14 (Fig. 1). The 5′ terminus of the RNA appeared to be several hundred base pairs inside the end of the DNA homology region, whereas the 3′ terminus [exclusive of poly(A)] was quite close to the opposite boundary.

Terminal repeated sequences in λMIA14 and other A-particle genes. A 1.1-kb *Hin*dIII fragment and a 1.8-kb *Eco*RI fragment encompassing the 3′ and 5′ ends, respectively, of the A-particle gene in λMIA14 (Fig. 1) were individually cloned into pBR322, and recombinants containing inserts in the same orientation were selected (pMIA6 and pMIA7, respectively). The cloned 3′ insert, when reisolated and labeled by

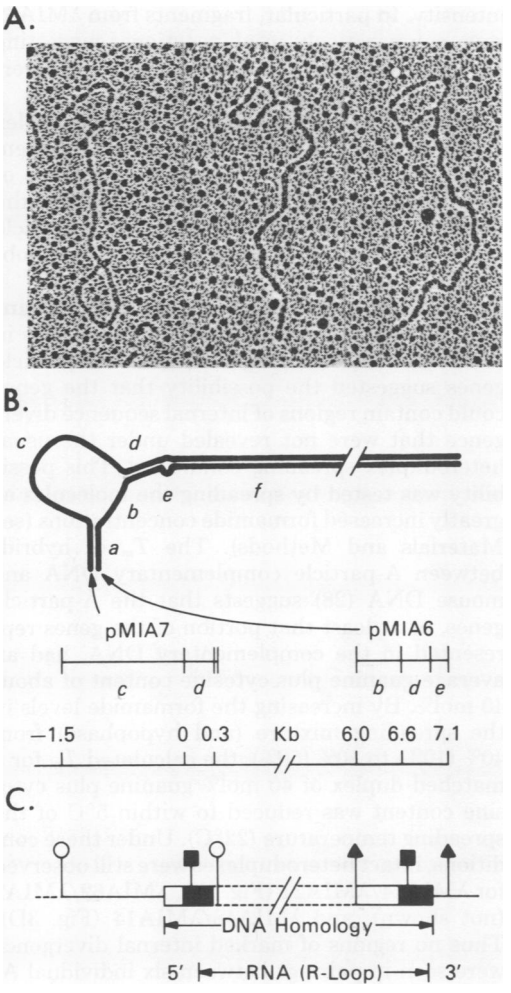

FIG. 4. *Size and position of terminally repeated sequences in the 7.3-kb A-particle gene in λMIA14. (A) Examples of heteroduplexes formed between recombinant plasmids pMIA7 and pMIA6 which contain restriction fragments from the 5′ and 3′ ends of the λMIA14 gene, respectively. In pMIA7 the fragment was inserted at the EcoRI site of pBR322 (map position 0); in pMIA6 the fragment was inserted at the HindIII site (map position 29). For heteroduplexing, the plasmids were linearized by cutting at the pBR322 BamHI site (map position 375). (B) Diagrammatic representation of the heteroduplexes. Region a represents pBR322 sequences between the BamHI and HindIII sites, measured size of 360 ± 60 bp (346 bp expected); region f is the remainder of the pBR322 sequences, measured size of 3,900 ± 130 bp (3,985 bp expected). Other regions are formed by the MIA14 inserts, shown with respect to their map positions in the λMIA14 gene: d represents the double-stranded segment formed by the terminally repeated sequences, size of 320 ± 60 bp. The single-stranded regions b and c measured 530 ± 100 bp and 1,580 ± 170 bp, respectively; e was too short to be estimated. (C) Placement of the terminally repeated segments with respect to the region of DNA homology and the position of the 35S RNA as previously determined by R-looping.*

nick translation, hybridized strongly with the 5′ fragment (not shown), indicating the presence of terminally redundant sequences. The redundancy was visualized directly in heteroduplexes between the two plasmid recombinants (Fig. 4); the length of the repeated segment was estimated as 320 ± 60 base pairs (bp). The dimensions of the heteroduplex were consistent with the model shown in Fig. 4, in which the repeated sequences span the *Pst*I sites at map positions 0 and 6.6.

At the 3′ end of the gene, the repeat segment appeared to be coterminal with the regions of DNA and RNA homology as determined by heteroduplex and R-loop analysis, respectively. At the 5′ end, the DNA homology regions appeared to extend about 300 bp beyond the repeat segment, but this difference may not be significant since it is within the combined errors of the various measurements. The 5′ terminus of the 35S RNA probably lies within the terminal repeat sequences, but the precise position remains to be established.

The cloned 3′ fragment from λMIA14 was used as a probe in blot hybridization experiments to test for related repeat sequences in other A-particle genes. Eight different genes were examined, and in each case reactions were obtained with restriction fragments derived from both the 3′- and 5′-terminal regions. Figure 5 shows data obtained for λMIA14 itself and the other three genes mapped in Fig. 1.

Very strong reactions were obtained with restriction fragments from the 3′ ends of all of the genes: these included a uniform 0.6-kb *Hin*dIII/*Pst*I segment and *Hin*dIII fragments ranging from about 0.75 to 1.1 kb. When the *Hin*dIII fragments were small, reactions were sometimes seen with fragments to their right (λMIA21 and λMIA62). The data were consistent with a set of common sequences ending at a map position near 6.8 (Fig. 1 and 5).

The 3′ probe gave weaker reactions with fragments from the 5′ ends of the genes, as expected from the fact that the terminally redundant segment represents only 30% (0.32 kb/1.1 kb) of the total probe sequence. In all cases, reactions were obtained with *Pst*I/*Hin*dIII fragments derived from either side of the *Pst*I site at map position 0, confirming that the repeated sequences span this site in other genes. The lack of hybridization with *Eco*RI or *Eco*RI/*Hin*dIII fragments to the right of map position 0.3 was also consistent with the location of the repeated segment as shown in Fig. 4. The reactions of the 3′ probe derived from MIA14 with the 5′ fragments from other genes were not all of equal

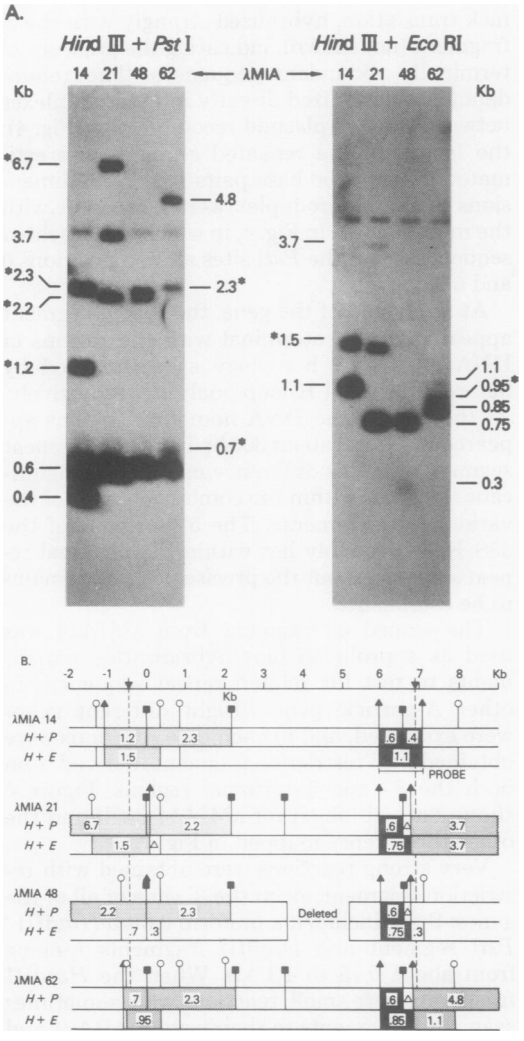

FIG. 5. *Hybridization of a 3′ probe against the HindIII/PstI and HindIII/EcoRI restriction fragments of λMIA14, -21, -48, and -62. A 1.1-kb HindIII restriction fragment representing the 3′ end of the A-particle gene in λMIA14 (Fig. 1) was cloned in pBR322; the recombinant plasmid (pMIA6) was cleaved with HindIII, and the fragments were* ^{32}P *labeled by nick translation. (A) Blot hybridization patterns after digestion with the indicated enzyme combinations. Fragments derived from the 5′ ends of the various λ recombinants are indicated by asterisks (*). The uniform light bands at 3.8 and 5.2 kb in the left and right panels, respectively, result from the reaction of probe with contaminating bacterial DNA. (B) A diagrammatic representation of the location of reactive fragments in the four λ recombinants. H, P, and E indicate the restriction enzymes HindIII, PstI, and EcoRI, respectively. Fragments marked with △ were 0.2 kb or less in size and were not included in the gels. In the H + E digest of λMIA48, the 3′ and 5′ fragments happen to be similar in size, so that identification of the labeled fragments in the blot of this digest was not possible. Arrows (↓) on size scale and dashed vertical lines indicate limits of DNA homology region as defined by heteroduplex analysis. Restriction sites are as shown in Fig. 1.*

intensity. In particular, fragments from λMIA62 gave comparatively weak reactions, suggesting an abbreviated 5′ redundancy region or divergent terminal sequences in this gene.

Unpaired DNA molecules in our heteroduplex mixtures were routinely scanned for loop-stem structures that might indicate the presence of extensive inverted repeat sequences in either the terminal or interior regions of the A-particle genes; in no instance were such structures observed.

Heteroduplex examination under stringent spreading conditions. The variations in restriction sites among individual A-particle genes suggested the possibility that the genes could contain regions of internal sequence divergence that were not revealed under the usual heteroduplex spreading conditions. This possibility was tested by spreading the molecules at greatly increased formamide concentrations (see Materials and Methods). The T_m of hybrids between A-particle complementary DNA and mouse DNA (28) suggests that the A-particle genes, or at least that portion of the genes represented in the complementary DNA, had an average guanine plus cytosine content of about 40 mol%. By increasing the formamide levels in the spreading mixture (and hypophase) from 40% (10%) to 80% (50%), the calculated T_m for a matched duplex of 40 mol% guanine plus cytosine content was reduced to within 5°C of the spreading temperature (22°C). Under these conditions, intact heteroduplexes were still observed for λMIA14/λMIA21 (Fig. 2F), λMIA62/λMIA2 (not shown), and λMIA48/λMIA14 (Fig. 3D). Thus no regions of marked internal divergence were seen in pairings between six individual A-particle genes.

A further increase in stringency equivalent to 7°C was effected by reduction in buffer concentration during spreading (see Materials and Methods). Under these conditions, where the calculated T_m for the matched duplex was 2°C below the spreading temperature, both hetero- and homoduplexes in the mixtures showed partial melting (data not shown).

Constancy of genomic restriction patterns in strains of *M. musculus*. The variation in occurrence of certain restriction sites (e.g., *Eco*RI and *Pst*I) divides the large family of A-particle genes into a number of subgroups which contribute to, and can be identified in, the complex restriction patterns derived from the whole mouse DNA (29).

Figure 6A shows the *Eco*RI/*Hin*dIII patterns obtained from liver DNA of 12 laboratory mouse strains, all inbred except NIH Swiss. The sample

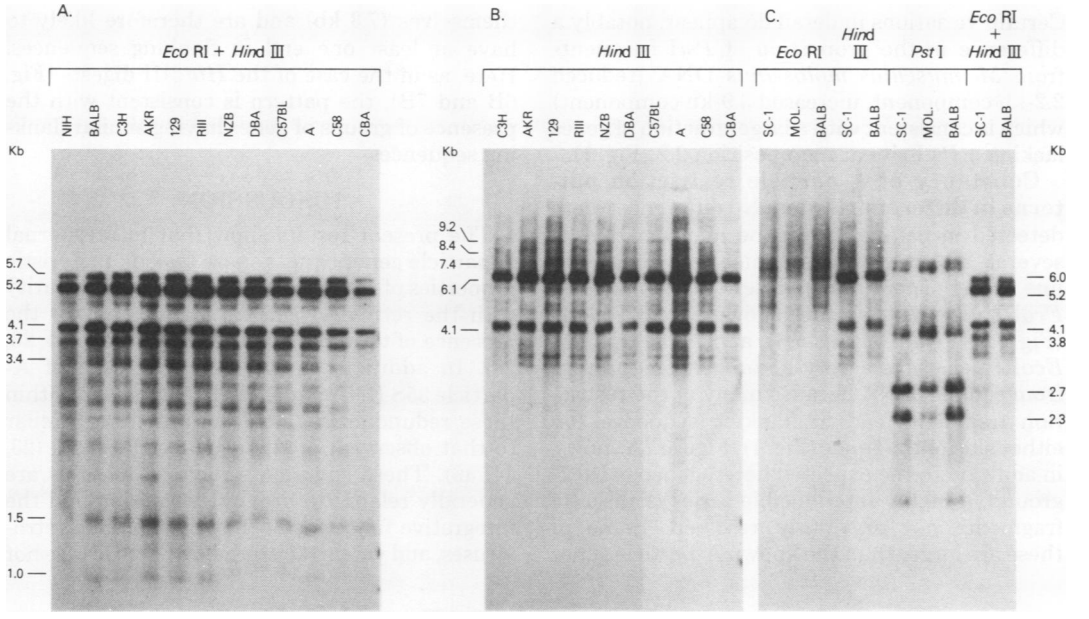

FIG. 6. *Restriction enzyme patterns of nuclear DNA from laboratory and feral strains of M. musculus. Electrophoretically fractionated digests (representing about 4 μg of DNA) were transferred to nitrocellulose membranes and hybridized with ^{32}P-labeled 35S A-particle RNA except where indicated. (A) Liver DNA of laboratory strains, digested with EcoRI plus HindIII. (B) Liver DNA of laboratory strains, digested with HindIII alone. The blot was hybridized with [^{32}P]RNA, and autoradiographs were prepared. The blot was then stored until no bands could be detected on an overnight autoradiographic exposure and was rehybridized with ^{32}P-labeled cloned A-particle DNA from λMIA14 (see the text). A second autoradiograph was then prepared. In (B) the first six lanes show the reaction with RNA, and the following four lanes show the reaction with cloned DNA to illustrate the identical reactions obtained with the two types of probe. (C) Comparison of BALB/c liver DNA With the DNA of SC-1 cultured cells and the liver of M. musculus molossinus (MOL), two examples of feral mouse cells.*

includes two strains, NZB and RIII, developed in other countries, and several domestic lines which have been separately bred for more than 50 years (41). The conservation of the restriction pattern in every detail is striking. With the isolation of λMIA62 and λMIA48, individual genes have now been found which can account for the four major bands at 5.7, 5.2, 4.1, and 3.7 kb, as well as smaller fragments between 0.5 and 1.0 kb. A ladder of intermediate-sized fragments (1 to 3 kb) is still unexplained, although there is evidence that some may come from A-particle genes with additional internal *Eco*RI sites. In any event, the relative abundance of A-particle restriction site variants detected with this enzyme combination is essentially identical in all the mouse strains.

Sets of identical patterns were also obtained after digestion with *Eco*RI, *Pst*I (neither is shown), and *Hin*dIII (Fig. 6B). No difference was detected between blots probed with ^{32}P-labeled 35S RNA or cloned DNA representing a single gene, λMIA14 (Fig. 6B). *Hin*dIII generates a major 6-kb band from 7-kb genes which, like λMIA21, have a *Hin*dIII site near map position 0 (Fig. 1), and larger fragments from others, such as λMIA14 and λMIA62, in which the site is located in variable flanking sequences. This variation was reflected in previous genomic blots of myeloma DNA as heterogeneous material larger than 6 kb (29). It is evident in Fig. 6B, however, that a series of discrete fragments in the size range of 7 to 9 kb is superimposed on this heterogeneous background, suggesting that subsets of A-particle genes may have similar flanking sequences. A 4.1-kb component is also prominent in the *Hin*dIII digests of mouse DNA (Fig. 6B). The origin of this component is not clear; however, both it and the major 3.7-kb band seen in the *Eco*RI/*Hin*dIII patterns (Fig. 6A) could reflect the presence of multiple deleted genes of the type exemplified by λMIA48.

BALB/c liver DNA was compared with DNA of a tissue culture line, SC-1, derived from a feral mouse of the western United States, and the liver DNA of a feral Japanese subspecies, *M. musculus molossinus* (Fig. 6C). The patterns were similar in major aspects, showing that the general organization of A-particle genes is not peculiar to laboratory strains of *M. musculus*.

Certain variations in detail do appear, notably a difference in the proportion of *Pst*I fragments from *M. musculus molossinus* DNA (reduced 2.2-kb component, increased 3.9-kb component) which is consistent with a larger fraction of genes lacking a *Pst*I site at map position 2.2 (Fig. 1).

Constancy of A-particle restriction patterns in different tissues. No differences were detected in patterns from the nuclear DNA of several BALB/c tissues, which included germ line cells (spermatids) after digestion with *Eco*RI plus *Hin*dIII (not shown), *Eco*RI alone (Fig. 7A), *Hin*dIII (Fig. 7B), and *Pst*I (Fig. 7C). *Eco*RI generates a very heterogeneous pattern from cellular DNA because many of the restriction fragments end in flanking sequences on either side of the genes (Fig. 1). Figure 7A shows, in addition to the expected heterogeneous background, a highly reproducible series of discrete fragments not previously resolved. Some of these are larger than the known A-particle genes themselves (7.3 kb) and are therefore likely to have at least one end in flanking sequences. Here, as in the case of the *Hin*dIII digests (Fig. 6B and 7B), the pattern is consistent with the presence of groups of genes having similar flanking sequences.

DISCUSSION

The present results show that intracisternal A-particle genetic units share two characteristic properties of retroviral proviruses: (i) colinearity with the retroviral genomic RNA and (ii) the presence of terminal repeated sequences (14, 23, 24). In addition, the 5′ and 3′ termini of A-particle 35S RNA appear to be positioned within these redundant segments in a manner similar to that observed for the usual retroviruses (23, 44, 46). These common structural features are generally related to proviral formation and the integrative functions of the transmissible retroviruses, and yet intracisternal A-particles are not

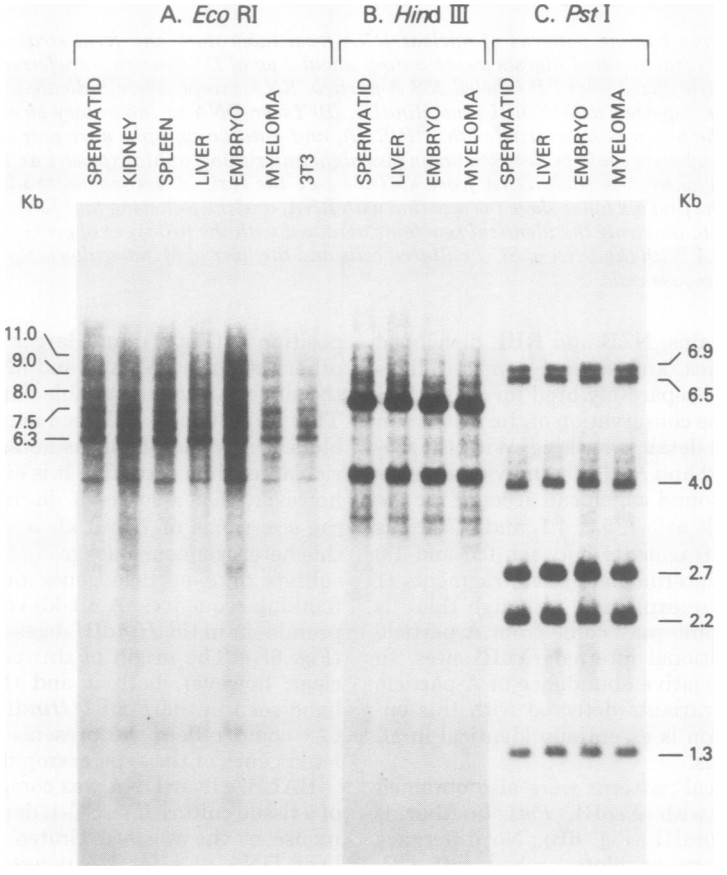

FIG. 7. *Restriction enzyme patterns of nuclear DNA from various tissues of a single inbred mouse strain, BALB/c. Digests of 4 μg of DNA were fractionated, transferred to nitrocellulose, and hybridized with ^{32}P-labeled A-particle 35S RNA. (A) Digestion with EcoRI. (B) Digestion with HindIII. (C) Digestion with PstI.*

known to have an infectious extracellular form. Sequences homologous to A-particle RNA, as well as an antigenically related structural protein, have indeed been found (26, 27) in a transmissible retrovirus, M432, derived from the species *Mus cervicolor* (9, 10), and it might be supposed that the intracisternal A-particle of *M. musculus* is simply an evolved defective equivalent of the M432 virus. However, a detailed structural comparison of the two types of genes in cloned form suggests that this is not the case, but rather that the M432 virus has acquired limited segments of A-particle sequence as if through recombination with A-particle genes endogenous to *M. cervicolor* (R. Callahan, K. K. Lueders, E. L. Kuff, and E. Birkenmeier, manuscript in preparation).

It is not known whether A-particle genetic components entered the mouse through infection with a once-competent retrovirus or whether they have evolved as cellular genes to their present retrovirus-like form. Homologous sequences in the DNAs of two Asian species, *M. cervicolor* and *Mus caroli*, show divergence commensurate with the evolutionary separation of these species from *M. musculus* (27); therefore A-particle sequences have existed within the members of this genus for some 4 to 5 million years (11, 37). During this period, there has developed a striking difference in A-particle gene copy number between *M. musculus* (500 to 1,000 copies per haploid genome) and the other two species (25 to 50 copies) (27). Comparison of A-particle sequence organization in *M. musculus* and the Asian mice could indicate the direction of evolution of this genetic element, since changes may have occurred more rapidly in the expanded gene pool. Temin and co-workers have postulated that retroviruses evolved from transposable elements through sequential acquisition and modification of the appropriate cellular genetic information (39). It will be important to determine whether A-particle homologous sequence elements in *M. cervicolor* or *M. caroli* contain terminal redundancies and whether both these and the repeated segments in *M. musculus* genes show the detailed structural features (e.g., inverted repeats or small flanking duplications of "host" sequences, or both) that are characteristic of transposable elements in lower organisms and the integrated avian and mammalian retroviral proviruses (12, 18, 19, 39, 44, 46).

Blots of *M. musculus* genomic DNA show that both the same basic organization of the A-particle genes and the same pattern of variation in restriction sites among these genes can be found in feral mice widely separated from the laboratory strains. The data thus far obtained indicate that the relative proportion of restriction site variants may differ among some subspecies of *M. musculus*, but are not subject to major short-term changes on inbreeding (50 to 60 years) or during tissue differentiation. The banding patterns provide some evidence for the presence of subsets of A-particle genetic units with similar flanking sequences, and in this aspect also, the patterns show a high degree of conservation within the laboratory strains. The relative stability of the A-particle patterns in *M. musculus* is consistent with their long-term evolution as a cellular genetic system (whatever their origin), and the observed conservation of homology between individual isolated genes suggests that some selective pressure may operate on this system.

Marked strain-specific differences in copy number and insertion sites have been found for endogenous mammary tumor virus proviral DNA (15, 20). In the case of the endogenous murine leukemia viruses, DNAs of different inbred mouse strains showed similar *Eco*RI restriction patterns, although each strain had minor characteristic differences (3, 42). DNA from the feral SC-1 cell line had a very different pattern, however, showing that the constancy of proviral patterns observed in laboratory mice is not a general property of *M. musculus* (42). The multiplicity of A-particle genetic units in this species makes it difficult to detect small changes in copy number or comparable differences in the insertion sites of individual genes.

A-particle genetic units make up one of the most reiterated sets of structural genes thus far observed in *M. musculus*. However, with the exception of the deletion found in λMIA48, no major sequence alterations or substitutions have yet been detected among the various isolated genes examined by heteroduplexing or R-looping in this or our previous study (29). In fact, for the limited sample examined under more stringent spreading conditions, the overall sequence conservation between gene pairs appeared to be good. Nevertheless, our experience is still small relative to the total number of A-particle genes, and certainly we have not observed the full range of variability in the population. Multiple samples of genes that code for a virus-like 30S RNA (22) have been isolated from a mouse gene library (25). These genes also showed base sequence differences reflected in variations in particular restriction sites; but in addition, they contained multiple internal regions of nonhomology up to 300 bp in length. These have not been seen in the sample of A-particle genes thus far examined.

Very recently, Ono et al. (35) reported on the sequence organization of some cloned intracisternal A-particle genetic elements. Using ^{125}I-

labeled poly(A) RNA from a mouse myeloma as a hybridization probe, the authors first selected reactive recombinants from a mouse gene library prepared by insertion of *Eco*RI-digested Swiss mouse DNA into λ Charon 4A. Inserted mouse DNA segments containing a group of common sequences were then transferred into pBR322 and subsequently identified as A-particle specific by virtue of their hybridization with a 3.5-kb poly(A) RNA isolated from myeloma-derived A-particles. The transcriptional orientation was determined by hybridization of restriction fragments with an oligodeoxythymidylic acid-primed complementary DNA probe. Seven plasmid recombinants, containing DNA inserts from 3.1 to 6.8 kb in size, were studied; five of the inserts had a set of common 3′ sequences about 3 kb in length. The 5′ regions of these five genes showed several types of variation, including major deletions and substitutions, and two other genes contained large central deletions. No genes containing a 7.3-kb region of DNA homology were observed.

The heterogeneity of this gene population is in striking contrast to what we have observed with the 7.3-kb A-particle genes. The common 3′ segments of the genes described by Ono et al. contain *Bam*HI and *Hin*dIII restriction sites corresponding to those seen at positions 3.9, 5.4, and 6.0 on the map of the larger genes (Fig. 1). However, the relation of the 5′ ends of the two groups of isolates is obscure. As noted previously (29) and in the present study, genomic blots of mouse DNA show a variety of reactive restriction fragments which cannot be readily explained on the basis of the 7.3-kb genes (e.g., the *Eco*RI/*Hin*dIII fragments between 3.4 and 1.5 kb in Fig. 5A), and it was suggested that shorter forms of the genes could be present. It would be interesting if two different methods of approach have selected different portions of the large population of A-particle-related genetic units. On the other hand, it remains to be determined whether some of the variant structures observed by Ono et al. may have arisen during the isolation procedure employed in their study.

Although A-particles are quite abundant in preimplantation mouse embryos (7, 8, 13) and in certain mouse tumor cells (30), we have no idea of the fraction of transcriptionally active genes in these situations or whether any such genes are included among our isolates. A-particles from different tumors contain, in addition to 35S RNA, a variety of smaller but sequence-related RNA species: e.g., a 32S RNA is abundant in particles from neuroblastoma and MOPC-21 myeloma cells, whereas a 29S form predominates in MOPC-104E (36). Clearly, these could represent transcripts of gene variants, or groups of variants, which had been differentially activated in the different cell types. These questions can perhaps be approached through detailed structural analysis of cloned genes and by the analysis of gene transcription in intact and reconstructed systems. A more fundamental problem is to determine whether A-particle genes have some general role in cellular physiology and whether amplification of these genes in *M. musculus* has a selective value or is evolutionarily irrelevant.

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