Terminally Redundant Sequences in Cellular Intracisternal A-Particle Genes

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The sequences coding for intracisternal A-particle RNA form a family of related but not identical genetic elements which are present in 650 to 1,000 copies within the mouse genome. We showed that different intracisternal A-particle genes had a terminally redundant sequence of about 400 base pairs, one-half of which arose from the 3' end of the intracisternal A-particle RNA. A second portion of the redundant region did not contain 3'-related sequences and was probably derived from the 5' end of intracisternal A-particle RNA. Thus, there were endogenous intracisternal A-particle genes in the cellular DNA-3'-5'—3'-5'-cellular DNA configuration identified for type B and C retroviruses. This indicated that the initial integration of intracisternal A-particle genes into the Mus musculus genome occurred by the same mechanism as the integration of other retroviruses. Two types of heterogeneity were identified among the 5' sequences of the two genes.

Intracisternal A particles are retrovirus-like structures that are found budding from the endoplasmic reticulum of many transformed mouse cells (7, 9, 12, 16) and during very early stages of normal embryogenesis (1, 3, 5). There are approximately 1,000 copies of the sequences coding for intracisternal A-particle RNA (21, 26), and these genes appear to be interspersed throughout the mouse chromosomes (21). Previously, we have shown that individual intracisternal A-particle genes share common sequences, yet can differ substantially in overall sequence content and organization (26). Cloned A-particle genes have also been analyzed by Lueders and Kuff (23), who described many genes similar to gene 81-A described here. Thus, genes represent a diverse family of genetic elements that occupy over 0.2% of the mouse genome. It is important to understand the origin of this large number of genes and what factors govern their expression in transformed, as well as normal, cells. Isolated intracisternal A-particles are not infective when injected into mice (14, 20), so the relationship of these particles to transmissible retroviruses has remained uncertain. There appears to be no sequence homology between intracisternal A-particle genes and type B and C retroviruses (22, 32), but some sequence homology between intracisternal A-particle genes and a transmissible retrovirus from Mus cervicolor has been observed (19).

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We have isolated several bacteriophage lambda recombinant DNA clones containing newborn mouse DNAs that contain intracisternal A-particle genes, and the sequence organization of these genes has been reported previously (26). In the work described here, we identified long terminally redundant sequences of 400 base pairs in two of the cloned genes and found that these redundancies contained both 3' and 5' sequences from intracisternal A-particle RNA. Sequences of this form, which are somewhat similar to the direct repeats of bacterial transposable elements (2), have been implicated in the replication and integration of type B and C retroviruses (13, 15, 29). Furthermore, we found two different levels of sequence heterogeneity in the cloned genes. Identification of the redundant sequences provided strong evidence that intracisternal A-particle genes are exogenous retroviral genomes that have integrated into the host genome by the same mechanism as other retroviruses, even though intracisternal A-particle genes are present in over 20 times as many copies as any other type.

MATERIALS AND METHODS

Restriction enzymes were purchased from Bethesda Research Laboratories, and digestions were performed according to the instructions of the supplier. DNA fragments were electrophoresed in a 1.5% agarose gel (25) and transferred to nitrocellulose filters by the method of Southern (31). Nick translation was carried out by the method of Rigby et al. (28), using DNA fragments isolated as previously described (26). Characterization of the cloned DNA, isolation of intracisternal A-particle RNA, and complementary DNA syn-
thesis were as previously described (26). Filter hybridization was performed in a solution containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), 0.02 M sodium phosphate (pH 6.8), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.1% sodium dodecyl sulfate (10) for 16 to 18 h at 65°C. Filters were washed four times at 65°C with 3× SSC−0.1% sodium dodecyl sulfate and two times at 65°C with 0.1× SSC−0.1% sodium dodecyl sulfate unless otherwise noted. Heteroduplex studies were performed by the method of Davis et al. (8), using a spreading buffer containing 50% formamide, 0.1 M Tris-hydrochloride (pH 8.5), and 0.01 M EDTA. The hypophase contained 20% formamide, 0.01 M Tris-hydrochloride (pH 8.5), and 0.001 M EDTA; 15 to 20 molecules of each type were measured. The lengths (in kilobases) were determined from double- and single-stranded fragments on the same grid, which were of known length from gel electrophoresis (24).

RESULTS

In a previous report, we described the isolation and characterization of several bacteriophage CH4A recombinant DNA clones containing endogenous murine intracisternal A-particle genes inserted into EcoRI sites (26). Two of these clones (clones 19 and 81) contain multiple EcoRI fragments that hybridize to A-particle RNA, and these clones were selected for further study. The order of the EcoRI fragments within each clone was determined initially by partial restriction enzyme digestion. The tentative map was verified by subcloning each of the EcoRI fragments into pBR322, determining other restriction enzyme sites within each fragment, and showing that the fragment sizes predicted for digestion of the λ clones with these other enzymes were in fact present (Fig. 1). For each of the two isolates, the order of the EcoRI fragments is shown in line 1 (Fig. 1), a more detailed restriction enzyme map is shown in line 2, and the gene region is shown in line 3. Each gene region was identified by homologous regions in heteroduplexes between the cloned genes (26) and by hybridization of labeled intracisternal A-particle RNA to the same contiguous blocks of sequences but not to the flanking regions (data not shown). The terminally redundant regions described below are indicated by the small boxes at the ends of each gene region in line 3 (Fig. 1). Gene 81 corresponds to the major class of intracisternal A-particle genes described recently by Lueders and Kuff (23). Gene 19 corresponds to another of the major bands of A-particle genes evident in mouse DNA digested with EcoRI plus HindIII (23; unpublished data).

The restriction enzyme maps show that there was a PstI site at each end of both genes and 0.64 kilobase (kb) from a conserved HindIII site at the right end of each gene. The similarity of the sequences around the common PstI sites was investigated by isolating a small fragment from the right end of clone 81 (Fig. 1, fragment a) and hybridizing it to individually subcloned EcoRI fragments containing the ends of the clone 81 gene (fragments A and C [Fig. 1A, line 1]) and both ends of the clone 19 gene (fragments B and D [Fig. 1B, line 1]). The subcloned fragments were digested with EcoRI, PstI, and HindIII to subdivide these sequences further. After electrophoresis on an agarose gel, the frag-

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Fig. 1. Restriction enzyme maps of two intracisternal A-particle genes. For each clone, line 1 shows the order and sizes (in kilobases) of the different EcoRI fragments. Line 2 shows a more detailed restriction enzyme map (EcoRI [1], BamHI [2], HindIII [3], and PstI [4]). Line 3 indicates the gene region in each case. The sequence homology between the two genes and the gene organization within the clone 81 fragment A and clone 19 fragment B regions have been described previously (26). The boxes in line 3 show the positions of the terminally redundant regions containing sequences from both the 3' end and the 5' end of A-particle RNA.
ments were transferred to nitrocellulose filters by the method of Southern (31) and hybridized to a nick-translated probe of fragment a. Figure 2b shows that this fragment hybridized strongly to the DNA at the left side of the PstI site at both ends of both genes, indicating that the same sequences were present. Also evident was the hybridization to a partially digested DNA fragment of 3.9 kb in clone 19 fragment B.

Previously, we mapped the 3' end of the major intracisternal A-particle RNA from mouse plasmacytomas to a point near the PstI site at the right side of the gene region (26). We wished to determine whether the 3' sequences in the RNA were present at both ends of the integrated genes. A probe specific for the 3' sequences of A-particle RNA was synthesized by using an oligodeoxynucleotide primer and a reverse transcriptase. The average length of this complementary DNA was only 400 nucleotides. Figure 2c shows that the 3' probe hybridized to the same fragments as the fragment a probe described above. In addition, we again found hybridization to the 3.9-kb partial digestion fragment in clone 19 fragment B, as well as to another fragment at 3.6 kb. This latter band may represent some internal sequence homology to the ends of the gene or possibly an internal primer of complementary DNA synthesis. Thus, the 3' end of A-particle RNA was duplicated in the endogenous genes in a structure analogous to the proviruses of other murine and avian retroviruses (13, 15, 29, 30). The 3' duplication did not seem to extend to the right side of the PstI site to any detectable extent.

This duplication of 3' sequences suggested that the 5' sequences of A-particle RNA might also be redundant. A small DNA fragment to the right of the PstI site in the clone 19 gene was isolated (Fig. 1, fragment b). This fragment did not contain 3' sequences, as indicated by the lack of hybridization to 32P-labeled complementary DNA. Recent S1 nuclease mapping data have shown that the sequences of the 5' end of A-particle RNA are within this fragment (Ono, Cole, and Huang, manuscript in preparation). This 5' fragment was hybridized to both ends of the genes, as described above for fragment a. Figure 3a shows that under the same hybridization and washing conditions, this probe hybridized to the two ends of the clone 19 gene, but not to either end of the clone 81 gene.

![Figure 2](http://jvi.asm.org/)

**Fig. 2.** Terminally redundant 3' sequences in intracisternal A-particle genes. The individual EcoRI fragments containing the ends of the two intracisternal A-particle genes were subcloned into pBR322 and digested with a combination of three restriction enzymes (EcoRI, HindIII, and PstI), generating the subfragments shown in Fig. 1, lines 2. (a) Stained fragments after electrophoresis in a 1.5% agarose gel, in which the 3.6- and 0.75-kb fragments common to all of the subclones were derived from the plasmid vector. (b) The 0.42-kb fragment from clone 81 fragment A (Fig. 1, fragment a) was isolated, nick translated, and hybridized to a Southern blot of the gel in (a). The 3.9-kb band of hybridization in clone 19 fragment B arose from a partial enzyme digestion fragment evident in the stained gel. (c) An identical Southern blot was hybridized to short complementary cDNA prepared from MOPC 315 intracisternal A-particle RNA that represented the 3' sequences. This complementary cDNA was only 400 nucleotides long, as determined by electrophoresis on alkaline agarose gels (24).
However, when the stringency of washing was lowered from 0.1x SSC to 3x SSC (Fig. 3b), there was hybridization to the corresponding segments of the clone 81 gene. The other weak bands of hybridization in clone 19 fragment D and clone 81 fragment C may have represented some weak homology of fragment b to DNA sequences outside the gene regions. Thus, there was a duplication of both the 5' and the 3' sequences of intracisternal A-particle RNA in the integrated genes, but there was a significant amount of sequence divergence in the 5' sequences that was not found in the 3' region.

The size of the terminally redundant region and its position with respect to the restriction enzyme sites were determined by electron microscopy of heteroduplex molecules. The four EcoRI restriction fragments containing the ends of the genes were subcloned into pBR322 in such a way that when the circular plasmids were digested with BamHI, the molecules were linearized, but the terminally redundant regions remained linked to the pBR322. This approach was used to help stabilize the short region of homology in the heteroduplexes by allowing the vector DNAs to hybridize. This also identified more precisely the EcoRI sites at the boundary between the mouse and vector DNAs.

The heteroduplex formed between the two ends of the clone 81 gene is shown in Fig. 4a. There was a clearly recognizable duplex of approximately 400 base pairs arising from the terminally redundant sequences. A comparison of the restriction endonuclease maps and the heteroduplexes placed the PstI sites in the middle of the duplex, which was consistent with the finding described above that sequences on both sides of that site had been duplicated. There was no sequence mismatch evident, and there was no hybridization in the regions flanking the terminal redundancy. This region was smaller than the 0.57-kb region found in Moloney murine leukemia virus (13) or the recently sequenced spleen necrosis virus redundancies (30). When the left end of the clone 81 gene was hybridized to the right end of the clone 19 gene, a similar heteroduplex was observed (Fig. 4b). The sequences on both sides of the PstI site formed a stable duplex, as determined by electron microscopy, and there is no evidence of the sequence divergence demonstrated in Fig. 3.

An additional level of heterogeneity distinct from the 5' heterogeneity described above was found when the left end of the clone 19 gene was allowed to form a heteroduplex with the right end of the clone 81 gene. Figure 4c shows a heteroduplex between clone 19 fragment D and clone 81 fragment A. The same duplex region of 400 base pairs was evident, but there was a small, reproducible bubble representing approximately 100 unpaired bases in one of the genes. These unpaired sequences could be assigned to the left end of the clone 19 gene because the bubble was observed whenever that fragment was combined with the other fragments, including the opposite end of the same gene (clone 19 fragment B) (Fig. 4d). There was no apparent insertion or substitution of sequences in the right end of clone 19 fragment B since no unpaired bases were evident in heteroduplexes with either the left end (Fig. 4b) or right end (Fig. 4e) of the clone 81 gene.

There was an additional difference between the clone 19 and 81 genes, namely the distance from the left terminal repeat to the EcoRI site. In clone 81 fragment C, this distance was 200 base pairs (Fig. 4a and b). However, in clone 19 fragment D, the terminal repeat was immediately adjacent to the EcoRI site (Fig. 4c and d). Presumably, this change fell in a coding region in the retroviral gene itself. Thus, clone 19 fragment D could contain an insertion, yet still have about the same PstI-to-EcoRI fragment length (0.38 versus 0.35 kilobase pairs).
FIG. 4. Heteroduplexes of the individually subcloned ends of intracisternal A-particle genes demonstrating the terminally redundant 0.4-kb sequences. The EcoRI fragments shown in Fig. 1 that contained the ends of the intracisternal A-particle genes were subcloned into pBR322 so that the redundant sequences were closer to the single PstI site and further from the BamHI site in the vector. The plasmids were then digested with BamHI to linearize the molecules but maintain the linkage of the redundant sequence to the vector DNA. Some internal portions of clone 81 fragment A and clone 19 fragment B that were flanked by BamHI sites were excised. A diagram is presented beneath each micrograph to show the length of each combination (in kilobases) and the approximate positions of the restriction enzyme sites. The standard deviations for the lengths were from 5 to 15%, with the latter figure applying to the smallest lengths. (b) Arrow shows the single-stranded bubble and the EcoRI site. (c) Arrow indicates the small unpaired sequences from the 5' end of the clone 19 gene. (d) Arrow indicates the EcoRI site at the boundary between mouse DNA and pBR322.

The terminally redundant sequences in intracisternal A-particle genes are diagrammed in Fig. 5, which shows the approximate positions of the PstI and EcoRI sites and the positions of the inserted sequences at the left end of the clone 19 gene. A more detailed study of the positions of
the 5′ and 3′ sequences of the RNA within this redundant region will be presented elsewhere (Ono et al., manuscript in preparation).

**DISCUSSION**

We found that the intracisternal A-particle genes each have 400-base pair direct repeats at their ends. More detailed analyses of these redundant sequences indicated that both the 5′ and the 3′ sequences from A-particle RNA are duplicated; these duplications are analogous to the redundant regions described for the provirus of other murine and avian retroviruses (13, 15, 29, 30). This finding indicates that at least the initial introduction of A-particle sequences into the mouse genome occurred by a mechanism of replication and integration similar to the mechanism in type B and C viruses. In the models of retrovirus integration which have been described, reverse transcriptase begins at the tRNA primer near the 5′ end of the viral RNA and synthesizes a double-stranded DNA copy, duplicating the sequences at the 5′ and 3′ ends in the process (6, 13). We have identified duplications of both the 5′ and 3′ sequences at the ends of two different intracisternal A-particle genes, indicating that the endogenous sequences are of the form 3′-5′—5′-3′. A tRNA-primed reaction has not been demonstrated yet. Even though there is no homology between A-particle genes and type B and C retroviruses, the mechanism of establishing a proviral genome appears to be the same for all three.

In a previous study (26), we found that two different conserved segments of the intracisternal A-particle genes were present in different numbers within the mouse genome (650 copies of the 1.4-kb BamHI fragment and 1,800 copies of a 3-terminal fragment). This difference did not seem to be attributable to the small fraction of genes that lack the BamHI sequences. Our finding of redundant sequences in intracisternal A-particle genes indicates that there are about 900 copies, all with a terminal redundancy and most with the BamHI sequences.

We still do not know the origin of the large number of intracisternal A-particle genes within the mouse genome and which gene or genes are transcriptionally active in mouse myeloma. A single infection or many smaller infections of replication-competent A-particle viruses may lead to the numerous integrated genes found in the germ line DNA. However, no other retroviral sequences occur in more than 50 copies per haploid genome. A second possibility has been suggested by a comparison of the direct terminal repeats of these retroviral genes with transposable elements, both procaryotic (e.g., Tn9 [18]) and eucaryotic (the copia gene families of Drosophila [11] and the Ty1 element of yeast [4]). In the latter case, 300-base pair direct repeats are present at the ends of each gene (11), and the genes are both mobile within the genome and capable of dramatic changes in the number of copies (27). The same cellular mechanism responsible for these changes might act on endogenous retroviral genes as well.

In a previous report, we demonstrated that many of the sequences from the left half of the clone 81 gene were not present in the clone 19 gene, indicating that these two related A-particle genes are different. We show here that these differences extend to a divergence in the sequences that presumably encode the 5′ end of intracisternal A-particle RNA (Fig. 3a). This is in contrast to the sequences from the 3′ end of the RNA, which appear to be conserved between the two genes (Fig. 2). If these two genes have a common ancestor, one portion of the gene has been conserved much more tightly than the other. These differences may be the result of extensive recombinational events between members of a large family of intracisternal A-particle sequences. Another retroviral gene family from mice that is not related to A-particles may have undergone similar rearrangements (17).

The small 100-base pair insertion into the 5′ end of the clone 19 gene probably occurred after the integration event, because the same insertion is not present at the 3′ terminally redundant region of this gene. The exact sequence organization of the redundant regions is being analyzed by DNA sequencing. In this respect, we have found recently that sequences around the PstI sites of the clone 19 gene are the same at both ends (A. Brown and R. C. C. Huang, manuscript in preparation). These studies, combined with our previous examination of sequence organiza-
tion, demonstrate that intracisternal A-particle genes are highly reiterated and highly variable genetic elements within the mouse genome. Specific DNA fragments spanning all of the clone 81 gene EcoRI sites have been detected in total mouse DNA (Cole, Ono, and Huang, manuscript in preparation).

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LITERATURE CITED