Intracisternal A-Particle Genes: Structure of Adjacent Genes and Mapping of the Boundaries of the Transcriptional Unit

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Adjacent intracisternal A-particle (IAP) genes were identified in two different recombinant DNA clones, λ 81 and λ 19. In clone λ 81, the most common form of IAP gene was separated by 5.3 kilobases from another IAP gene that had two apparent internal deletions. The two genes were in a head-to-tail configuration. In clone λ 19, two different types of IAP genes were separated by less than 0.5 kilobase. Blot hybridization analysis of mouse DNA demonstrated that the DNA sequence found in clone λ 81 is identical to the *in vivo* configuration. Using isolated DNA fragments from clone λ 19, we mapped the boundaries of the IAP RNA by S1 digestion of RNA-DNA hybrids and by cDNA extension. With these techniques, both the 5' end and the 3' end of the IAP RNA in two different plasmacytomas (MOPC 315 and TEPC 15) were shown to fall within the long terminal direct repeat of the IAP gene. The fragment sizes generated by S1 digestion of IAP RNAs isolated from the two tumor lines were found to differ, indicating that different IAP genes may be transcribed in these two plasmacytomas.

Intracisternal A-particles (IAPs) are retrovirus-like structures found budding from the endoplasmic reticulum of every mouse plasmacytoma examined to date (5). These particles contain a reverse transcriptase distinct from the C-type retrovirus enzyme (29) and a specific polyadenylated RNA that can represent as much as 5% of the total polyadenylated RNA in plasmacytomas (15, 30). Reassociation kinetic studies with both IAP cDNA (13) and cloned mouse DNA segments containing endogenous IAP sequences (17) indicate that there are approximately 1,000 IAP genes integrated into the mouse genome.

Genomic RNAs of retroviruses have short, direct repeat sequences at their 5' and 3' ends which are 21 nucleotides long in avian sarcoma virus (7, 22, 25) and 60 nucleotides long in murine leukemia virus (3). These sequences are thought to play an important role in the reverse transcription of the viral RNA. Both double-stranded circular and integrated forms of retroviruses have a much longer terminal redundancy (LTR) which includes the strong stop sequences and a portion of the 3' end of the RNA (8, 10, 20, 23). The ends of the genomic RNA thus map to the middle of the LTRs, and these sequences appear to be involved in integration as well as initiation and termination of transcription.

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In this paper, two recombinant DNA clones that contained adjacent IAP genes were analyzed. In one, IAP genes with different sequence organizations were found to be separated by 5.3 kilobases (kb), and the same configuration was shown to exist *in vivo*. We have previously suggested that the ends of the IAP RNA fall within the LTR sequence (4, 17). To study this further, S1 nuclease (2) and cDNA extension (28) mapping were used to position the 5' and 3' ends more precisely.

MATERIALS AND METHODS

Preparation of RNA. Mouse myeloma tumors were obtained from H. Eisen through Bionetics, Inc. (Rockville, Md.) and propagated subcutaneously. Total polyadenylated RNA was extracted from solid tumors as described previously (17).

Recombinant bacteriophage and plasmids. Recombinant DNA clones containing IAP genes have been described previously (4, 17). All operations were carried out in accordance with National Institutes of Health guidelines. Plasmid DNA was prepared by the method of Schibler et al. (21).

Electron microscopy of heteroduplex molecules was performed as described previously (17). Nick translation was carried out by the method of Rigby et al. (18). For the analysis of total mouse DNA, 10 μ g was digested with the indicated restriction enzyme, electrophoresed in a 0.6% agarose gel, and transferred to nitrocellulose by the method of Southern (24). The nitrocellulose filter was prehybridized in 50% formamide-5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.05 M sodium phosphate (pH 7.5)-

1× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) (6)–100 µg of heat-denatured, sheared calf thymus DNA per ml at 42°C for 24 h. This solution was then replaced with 5 ml of the same solution to which had been added 3×10^6 cpm of heat-denatured, nick-translated probe. Hybridization was at 42°C for 66 h followed by one 30-min wash in 1× Denhardt solution–2× SSC at 22°C and two 45-min washes in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C. The filter was dried and exposed to Kodak XAR film with a Du Pont Lightning Plus intensifying screen (27).

End labeling of DNA. Restriction enzymes were purchased from Bethesda Research Laboratories (Rockville, Md.). A 1.1-kb *Hind*III DNA fragment was isolated from subcloned plasmid 19B and 3' end labeled to position the 3' end of IAP RNA. The 3' ends of the DNA were labeled by using the Klenow fragment (11) of DNA polymerase I (New England Biolabs) as described previously (19). After end labeling, the DNA was purified by phenol extraction and ethanol precipitation. The specific activity of the end-labeled DNA was usually 5×10^5 cpm/pmol of ends. The 5' ends were labeled with T_4 polynucleotide kinase by the method of Maxam and Gilbert (16).

DNA-RNA hybridization and S1 nuclease digestion. Fifty micrograms of total polyadenylated RNA was dissolved in 50 µl of 10 mM Tris-hydrochloride (pH 7.5)–10 mM NaCl–1 mM EDTA, and end-labeled DNA (0.02 to 0.1 pmol) was added along with 40 µl of deionized formamide. The solution was then heated to 70°C for 10 min. After the addition of 5 µl of 3 M NaCl–0.3 M sodium citrate, hybridization was carried out at 45°C for 4 h. S1 nuclease (Sigma Chemical Co.)

digestion was performed as described previously (28). After digestion, nucleic acids were precipitated by ethanol, dissolved in 20 µl of 5 M urea–0.1 M NaOH–5 mM EDTA–0.0025% xylene cyanol–0.0025% bromophenol blue, heated to 90°C for 2 min, and analyzed on a 6% polyacrylamide–8 M urea gel containing 0.1 M Tris-borate (pH 8.3)–2 mM EDTA. After electrophoresis, the gel was autoradiographed with Kodak XR film for 1 to 2 days.

Reverse transcription. The 0.46-kilobase-pair *Eco*RI–*Sst*I fragment of plasmid 19-B (Fig. 1, fragment II) was end labeled as described above and used as a primer for reverse transcription. A 0.5-µg sample of labeled primer was hybridized with 50 µg of total polyadenylated RNA as described above, ethanol precipitated, and dissolved in 100 µl of 50 mM Tris-hydrochloride (pH 7.5)–50 mM KCl–10 mM MgCl₂–10 mM dithiothreitol–0.2 mM each dATP, dCTP, dGTP, and TTP, and 36 U of reverse transcriptase (a gift of J. Beard) was added. Incubation was performed at 37°C for 1 h; 5 µl of the sample was then mixed with 15 µl of 6.7 M urea–0.13 M NaOH–6.7 M EDTA–0.003% xylene cyanol–0.003% bromophenol blue and analyzed on a 6% polyacrylamide–8 M urea gel followed by autoradiography.

RESULTS

Two independently isolated recombinant DNA clones, designated λ81 and λ19, containing segments of Swiss mouse DNA have been described previously (17). In each of these clones, there are several restriction enzyme fragments

FIG. 1. Adjacent IAP genes. Restriction enzyme maps of two recombinant DNA clones containing IAP genes are shown, with the *Eco*RI fragments indicated above each map. The sequence organization of the different IAP genes was determined previously (4, 17) and is described in the text. Areas of similar shading represent homologous sequences as determined by electron microscopy and cross-hybridization. Predicted restriction enzyme fragments for *Sst*I, *Pst*I, and *Eco*RI and the probe used to detect these fragments in Fig. 4 are shown below clone 81. The position of the fragments used for S1 mapping (I) and cDNA extension (II) are shown below clone 19-B.

that hybridize to IAP RNA. It was subsequently found that each clone contained two different IAP genes, giving rise to the multiple bands of hybridization. A summary of the sequence organization of these two clones is shown in Fig. 1. Areas of similar shading represent homologous sequences as determined previously (4, 17) and below. To map the adjacent genes a combination of heteroduplex analysis and restriction enzyme digestion was employed. The clones were digested with *EcoRI*, and the fragments were labeled A through E for λ 81 and A through D for λ 19 by order of decreasing size. Initially the *EcoRI* fragments hybridizing to IAP sequences were subcloned into pBR322, and several restriction enzyme sites were determined (4, 17). The order of the *EcoRI* fragments was then determined by partial *EcoRI* digestion and by demonstrating that restriction enzyme fragments for other enzymes predicted from the subcloned fragments were indeed present (data not shown).

The IAP genes within each subcloned fragment were then investigated by visualization of heteroduplex molecules in an electron microscope. The sequence organization and terminally redundant sequences in two of the genes have been described (4, 17). The 81-B fragment was found to have an IAP gene different from those mapped previously, as shown by heteroduplexes between 81-B and two other IAP genes, 81-A and 14 (Fig. 2 and reference 17). The sequence organization could be determined for 81-B since all regions annealed to sequences of the other genes. The left 0.5 kb of the gene was homologous to gene 14, whereas the rest was homologous to noncontiguous sequences of 81-A. Gene 81-B appears to have two DNA segments deleted when compared with 81A. Setting the *PstI* site near the 3'-5' sequence boundary within the terminal repeat as the 0 point (4), the missing sequences extend from 0.95 to 1.6 kb and from 2.2 to 5.75 kb. The end of the second apparent deletion occurs at the same site as the deletions in two other IAP genes (17). The IAP genes in 81-B and 81-A are separated by approximately 5.3 kb (Fig. 2).

In the second recombinant DNA clone, λ -19, IAP genes were found on *EcoRI* fragments 19-A and 19-B. The gene within 19-A is colinear with the 81-A gene above, up to the point where it is interrupted by the arm of the bacteriophage λ cloning vector (data not shown) at an *EcoRI* site that is common to many IAP genes (12). *EcoRI* fragment 19-B contains a second gene with a distinctly different sequence organization which has been described previously (17). This second type of IAP gene contains a sequence of 0.5 kb not found in the more abundant genes represented by 81-A and 19-A above. Moreover, the 19-B specific sequence is found in the predominant

IAP RNA in two different plasmacytoma lines (17). A terminally redundant sequence from the 19-B gene occurs on the adjacent 19-D fragment (Fig. 1).

The IAP genes in clone λ -19 are in a tail-to-tail configuration. A graphic illustration of these tandem IAP genomes was provided by visualization in the electron microscope of "snapback" molecules after denaturing the DNA and reannealing briefly (Fig. 3). The IAP sequences in this clone are in opposite orientation, and therefore the separated strands can self-anneal. The small segment of DNA separating the two genes appeared as a small single-stranded loop at the side of the duplex. The region of homology extends 3.5 kb to the point where the sequences diverge.

In both λ 19 and λ 81, an *EcoRI* site falls between the two genes. This could have resulted from the in vitro joining of the two fragments during construction of the gene library from a partial *EcoRI* digest of mouse DNA. With 1,000 IAP genes per haploid complement of DNA, there is a small probability that this could occur. To prove that adjacent proviruses exist in vivo, mouse DNA was analyzed for the configuration of restriction enzyme sites found in the λ 81 clone. It was necessary to use the unique sequences between the two IAP genes as a probe because the IAP sequences themselves are highly reiterated. Figure 1 shows that there is an *SstI* fragment of 8.7 kb which contains parts of both genes and spans *EcoRI* fragments B through A. A *PstI* fragment of 4.8 kb also spans the *EcoRI* B and C fragments. Figure 4 shows the hybridization of an intergene probe (81-C, *EcoRI-PstI*; Fig. 1) to Swiss mouse DNA digested with *SstI*, *PstI*, and *EcoRI*. All of the predicted fragments were found (Fig. 4). These different but related IAP genes thus have the same configuration in vivo as in the recombinant DNA clone. The same fragments were found in BALB/c mouse DNA (not shown), indicating that this location has been stable in the mouse genome during the recent past. It was not possible to analyze the λ 19 clone in the same way because of the extremely short distance separating the two genes.

Analysis of the 3' end of IAP RNA by S1 mapping. Electron microscopy of RNA-DNA hybrids indicated that the IAP RNA in plasmacytoma lines MOPC 104E and MOPC 315 is colinear with the 19-B gene, except for a 0.7-kb sequence that is missing in the RNA. The 19-B gene has an LTR at its end (4), and it was of interest to determine whether the ends of the IAP RNA fall within the LTR sequence. S1 nuclease mapping (2) was used to locate the 3' end of the predominant IAP RNA in MOPC 315 and in another plasmacytoma line, TEPC 15.

FIG. 2. Heteroduplexes between *Eco*RI-generated DNA fragments containing IAP gene 81-B and (A) gene 81-A and (B) gene 14 (17). The diagram indicates the relative positions of the regions of homology with areas of similar shading. Strand lengths were determined from measurements of single- and double-stranded pBR322 molecules on the same grid. Bar, 1 kb.

The latter tumor was found to have a predominant RNA nearly identical to that in MOPC 315 as determined by RNA gel blot analysis and by electron microscopy (Shen and Cole, unpublished data).

The 19-B fragment was subcloned into pBR322 in such a way that the conserved *Hind*III site within the IAP gene was close to the *Hind*III site in the plasmid vector (Fig. 2 in reference 17). Digestion of this plasmid with *Hind*III generated a 1.1-kb fragment which was isolated and labeled at the 3' termini (Fig. 1, fragment I). After denaturation, the fragment was hybridized to MOPC 315 polyadenylated

RNA, and the hybrids were digested with S1 nuclease. One end of the labeled DNA fragment contained a small segment (29 nucleotides) of pBR322 DNA (26), which cannot hybridize to the RNA and is digested with S1. Figure 5 shows that a 550-nucleotide protected DNA fragment was found, placing the 3' end of the major IAP RNA at least 550 nucleotides from the common *Hind*III site. The distance between the *Hind*III and *Pst*I sites is 620 nucleotides; therefore the end of the RNA is at most 70 nucleotides to the left of the *Pst*I site.

TEPC 15 polyadenylated RNA was analyzed in a similar set of hybridization and S1 digestion

FIG. 3. Self-annealing of the IAP sequences in clone 19, showing the small segment of DNA separating the two genes. Bar, 1 kb.

experiments, generating a 620-nucleotide DNA segment as a major band in addition to one at 550 nucleotides (Fig. 5). This 620-nucleotide band places the end of the RNA very near the *Pst*I site. To test if one of the bands was the result of an infrequently cleaved S1-sensitive site within the hybrid, the amount of S1 nuclease was doubled. The relative intensity of the bands did not change, indicating that the TEPC 15 myeloma tumor line has at least one additional species of RNA that MOPC 315 lacks. Furthermore, since the boundary of the LTR extends at least 170 nucleotides to the left of the *Pst*I site, the RNAs in both tumors map to within and almost in the middle of the LTR.

Several smaller bands were also evident (Fig. 5) which may represent minor species of RNA. Another band at 1,150 nucleotides was a self-annealed probe. It is not known whether the sizes of the bands reflect differences in the 3' end of the IAP RNAs or small sequence mismatches within the RNA-DNA hybrids that are cleaved by S1.

Mapping the 5' end of IAP RNA by cDNA

extension. We have used the cDNA extension (28) method to position the 5' end of the IAP RNA. Reverse transcriptase will recognize a DNA primer hybridized to an RNA template and synthesize a complementary DNA. If the primer is near the 5' end of the RNA, the enzyme should run off the end, leaving a discrete fragment that is the sum of the primer and the newly synthesized DNA. Since electron microscopic mapping concluded that the 5' end of the predominant IAP RNA in MOPC 315 was near the left *Eco*RI site in 19-B (17), a radioactivity labeled 0.46-kb *Eco*RI-*Sst*I DNA fragment from this region was used as a primer (Fig. 1, fragment II). After hybridization of the primer to RNAs from both MOPC 315 and TEPC 15 and DNA chain extension with reverse transcriptase, a cDNA product of 780 ± 50 nucleotides was found (Fig. 6). A cDNA of this length would position the 5' end of the RNAs 320 nucleotides to the left of the *Eco*RI site within the LTR, assuming the DNA sequence of the extended molecules is colinear with the gene. Since the distance from the *Pst*I to the *Eco*RI sites is 330

FIG. 4. Restriction enzyme fragments complementary to the region between two IAP genes. DNA from neonatal NIH Swiss mice was digested with the indicated enzyme, electrophoresed through a 0.6% agarose gel and transferred to nitrocellulose (24). The filter was then hybridized to the nick-translated probe from λ -81 shown in Fig. 1, and the predicted fragments were detected by autoradiography. The fragment of 7 kb in the *Pst*I lane was from partial digestion of the mouse DNA and was not found in other experiments.

nucleotides, the 5' end of the RNA falls very near the *Pst*I site. A diagram of these measurements is shown in Fig. 7.

DISCUSSION

Previous studies have found no sequence specificity for the integration sites of retroviruses, nor have endogenous proviruses been found to serve as homologous target sites for the integration of exogenous viral DNA (10, 20). Only in one case have tandem retroviruses been described in which a preexisting Rous sarcoma virus provirus was duplicated with no viral intermediate (9). Cells with this duplication may have had a selective advantage for growth in culture.

The adjacent IAP genes described in this paper differ from those described in the previous study in that the two IAP genes themselves are not the same. Furthermore, in λ 81 there is a 5.3-kb sequence separating the two. The IAP genes in λ 19 are also different from the Rous sarcoma virus genes in that the proviruses are in a tail-to-tail configuration.

There was no indication of how the IAP genes in λ 81 arose. No sequence homology or repeating restriction enzyme site pattern between different parts of the intergene region was observed, making a gene duplication event

FIG. 5. S1 mapping of the 3' end of IAP RNA. A 5' end-labeled, 1.1-kb, *Hind*III-restricted DNA fragment from 19-B (Fig. 1, fragment I) was hybridized to polyadenylated RNA from myeloma tumors, digested with S1 nuclease, denatured, and electrophoresed on a 6% polyacrylamide gel containing 8 M urea. (A) Marker pBR322 DNA digested with *Taq*I (26) and 5' end-labeled with polynucleotide kinase. The unmarked band in the *Taq*I lane appears to be a partial digestion fragment from the pBR322. (B) S1-resistant fragment after hybridization to MOPC 315 RNA. (C) S1-resistant fragments after hybridization to TEPC 15 RNA. (D) S1-resistant fragments after hybridization to TEPC 15 RNA and digestion with two times the amount of nuclease used in C.

FIG. 6. cDNA extension mapping of the 5' end of IAP RNA. The *EcoRI-SstI* fragment from the left side of 19-B (0.46 kb; Fig. 1, fragment II) was labeled at its 3' end, hybridized to total polyadenylated RNA from myeloma tumors, reverse transcribed, denatured, and electrophoresed on a 6% polyacrylamide gel containing 8 M urea. (A) *TaqI*-digested pBR322. (B) MOPC 315 polyadenylated RNA as a template. (C) TEPC 15 polyadenylated RNA as a template. (D) No template. (E) End-labeled primer only.

unlikely. Furthermore, the two proviruses are quite different from each other, although 81-B could have been derived from 81-A by two deletion events. IAP genes are highly reiterated in the mouse genome, but random integration would predict an average intergene spacing of 3,000 kb. It has been suggested recently that some IAP genes may have similar flanking sequences (12).

We have so far identified six different sequence organizations for IAP genes (this study and reference 17). One of these, the 81-A gene, has also been described by Kuff and co-workers (12, 15). They have also found a partially deleted gene (12) which is similar to clone 20, which we have previously studied (17). A significant fraction of the IAP genes in mice are apparently identical, but there are also a variety of forms that are not common enough to be detected by Southern blot hybridization of mouse DNA. The apparent deletion in 81-B occurs at the same site within the IAP genome as the deletions in two other variant genes, 62 and 20 (17). It remains to be determined whether this sequence is conducive to deletion events or whether it is in some way related to the genetic structure of the IAP genome.

The ends of the IAP RNA from two myeloma tumor lines map to within the LTR of gene 19-B. The diagram in Fig. 7 shows that both ends of the RNA can be positioned near the *PstI* site. Taking into account experimental error, the ends of the RNA may well overlap, giving a terminally redundant RNA analogous to that of other retroviruses (3, 7, 22).

In principle, S1 mapping can be used to determine both the 5' and 3' ends of the RNA transcripts on a DNA template, but only if there is complete sequence homology. Many IAP genes have lost conserved restriction enzyme sites (14) and may have diverged significantly in sequence. S1 nuclease may then recognize the differences between the sequence of the cloned gene and the sequence of the RNA transcript. Thus, the difference between the 550- and 620-nucleotide bands (Fig. 5) in the 3' end mapping of RNA from the two tumor lines may be due to a small sequence variation between the transcribed genes rather than a difference in the

FIG. 7. Possible ends of IAP RNA mapped on cloned gene 19. The 3' end of the RNA in MOPC 315 was positioned at least 550 nucleotides from the *HindIII* site, and it may extend to the same point as the longer TEPC 15 transcript if a sequence mismatch is recognized by S1. Both ends of the RNAs map very close to the *PstI* site near the middle of the LTR. The exact length of the LTR region and whether or not the 5' and 3' sequences overlap to give a terminally redundant RNA will require precise nucleotide sequence analysis. Abbreviations: P, *PstI* site; E, *EcoRI* site; S, *SstI* site; H, *HindIII* site.

position of the 3' end of the RNA. The same situation can also be true in positioning the 5' end of the RNA. Nevertheless, from this study, most of the IAP transcripts have both ends of the RNA within the LTRs. It is expected that the LTR will contain signals for the initiation and termination of transcription. Specific initiation of transcription within the LTR has been found for Rous and murine sarcoma viruses (1, 31). Determination of the nucleotide sequence of the LTR of IAP genes will allow a more thorough investigation into this point (Brown and Huang, manuscript in preparation).

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