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Presence and Transcription of Intracisternal A-Particle-Related Sequences in CHO Cells

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We have characterized sequences expressed in Chinese hamster ovary (CHO) cells which are related to the intracisternal A-particle (IAP) genes of mice and Syrian hamsters. Several cDNA clones homologous to Syrian hamster IAP probes have been isolated and used to evaluate the abundance and expression of these retroviruslike sequences. DNA blot analysis with homologous Chinese hamster IAP probes revealed that IAP-related sequences are present in CHO cell DNA at moderately repetitive levels (approximately 300 copies per haploid genome). Sequence analysis has revealed the existence of at least two distinct families of IAP-related sequences in CHO cell DNA. Family I sequences exhibit identical 4.5-kilobase-pair internal deletions relative to complete IAP genomes of mice or Syrian hamsters, but family II sequences showed no major sequence discontinuities relative to the IAP genes of other species. Both families are expressed as abundant cytoplasmic RNA in CHO cells, but only family II sequences produce abundant transcripts of a size consistent with that of a full-length IAP RNA. Intact *gag*, *pol*, or *env* open reading frames were not present in sequences of either family, although incomplete open reading frames spanning putative p27 and protease regions of IAP genes were observed.

Chinese hamster ovary (CHO) cells have been used extensively in mammalian cell genetic studies for a variety of reasons, including a relatively low number of chromosomes in the near-diploid range and ease of cultivation (30, 34, 39). Furthermore, CHO cells are well suited for the expression of high levels of exogenous proteins and have been used for the production of several biologically active proteins with pharmaceutical applications (3, 5, 27, 35). However, information regarding the presence and expression of retroviruslike sequences in these cells is limited. Electron microscopy has revealed the presence of both intracellular and budding retroviruslike particles in these cells (8, 16, 17, 21, 38), but little regarding the presence or expression of sequences capable of encoding such particles has been reported.

We have initiated an investigation of retroviruslike elements present in CHO cell DNA with the goal of elucidating the structure and potential function of these elements. In this report, we present results regarding our characterization of a class of sequences present in CHO cells which are closely related to elements encoding intracisternal A-particles (IAPs) in mice and Syrian hamsters. IAPs are noninfectious, intracellular, retroviruslike particles which are present in a variety of tumor cells as well as in early embryonic cells from many rodents including mice, rats, and Syrian hamsters (for a review, see reference 14). IAP-related sequences are interspersed throughout rodent genomes at moderately repetitive levels of 10^2 to 10^3 copies per haploid genome (19, 36). Representative full-length IAP elements from mouse and Syrian hamster genomes have been cloned and sequenced in their entirety, revealing that IAPs are evolutionarily closely related to mouse mammary tumor virus (MMTV) (a type B retrovirus) and simian type D retroviruses (14, 22, 26).

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Genetic elements with sequence homology to Syrian hamster IAP (SHIAP) and mouse IAP genomes have previously been detected in Chinese hamster DNA by using solution and blot hybridization techniques (18, 19, 33), although membrane-bound intracisternal particles reported to be the products of IAP genes in mice and Syrian hamsters have not been observed in CHO cells. Nevertheless, the intracytoplasmic particles observed in CHO cells (8, 16, 17, 21, 38) are morphologically similar to those described for the evolutionarily related B-type and D-type retroviruses (37) as well as the infectious M432 retrovirus of *Mus cervicolor* (1, 9). The M432 virus has not been classified but shares antigenic determinants and extensive genomic sequence homology with murine IAP particles (2, 12). Therefore, a potential coding relationship between IAP-related sequences and intracytoplasmic particles in CHO cells exists.

Because previous reports (18, 19) indicated that IAP-related sequences are present in multiple, heterogeneous copies in CHO cell DNA, we have focused our investigation on those sequences which are expressed as cytoplasmic poly(A)$^+$ RNA and, as such, are more likely to represent potential particle-encoding species. We have isolated several clones from a CHO cell cDNA library by using SHIAP sequences for hybridization screening and have used these homologous CHO cell IAP (CHIAP) probes to evaluate the abundance and expression of IAP sequences in CHO cells. Nucleotide sequence information from these clones has been used to determine their polypeptide-encoding capacity and to evaluate their potential for encoding functional virus particles.

MATERIALS AND METHODS

Origin and propagation of cells. All CHO cells used were derived from the parental dihydrofolate reductase-deficient CHO cell line, CHO-K1 DUXB11 (34). For convenience, we

refer to these cells by their abbreviated designation, CHO-DUX. These cells were originally derived from an ovarian biopsy of an adult Chinese hamster (*Cricetulus griseus*) (30) with subsequent mutagenesis and selection for the dihydrofolate reductase-deficient phenotype (39). The CHO-C2B cell line is a recombinant derivative of the CHO-DUX line which was stably transfected with an expression vector containing the genes for human tissue-type plasminogen activator and mouse dihydrofolate reductase (3, 28). The CHO-FR4 cell line was derived from the parental cell line by transfection with vector plasmid containing the gene for dihydrofolate reductase only. The mouse myeloma cell line X63-Ag8.653 (11) and the human diploid fibroblast cell line MRC-5 (10) were obtained from the American Type Culture Collection. All cells were tested and found to be free from mycoplasma contamination, using microbiological cultivation, biochemical analysis (Mycotect; GIBCO Laboratories), and fluorescence microscopy after Hoechst bisbenzimid staining.

All cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham F-12 nutrient mixture supplemented with 2 mM glutamine and 10% fetal bovine serum. CHO-DUX cells were also supplemented with 7.5 mg of glycine per liter, 4.77 mg of hypoxanthine per liter, and 0.73 mg of thymidine per liter.

Isolation of CHIAP cDNA clones. Cytoplasmic RNA was isolated from CHO-FR4 cells by phenol-chloroform extraction of 1.0% Nonidet P-40 cell supernatants, and poly(A)$^+$ RNA was selected by chromatography on oligo(dT) cellulose. Double-stranded cDNA was prepared from poly(A)$^+$ RNA with a cDNA synthesis kit (Amersham Corp.) utilizing reverse transcriptase and oligo(dT) as the primer. After blunt-end ligation of linkers, cDNA was ligated to arms of *Eco*RI-digested λgt10, packaged in vitro, and plated on *Escherichia coli* C600hfl−. IAP-homologous clones were identified by plaque hybridization on nitrocellulose filters, using low-stringency conditions (42°C, 25% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt solution, 0.05 M sodium phosphate, 10% dextran sulfate, and 0.1 mg of denatured salmon sperm DNA per ml). The probes used for screening were four cloned *Hin*dIII fragments (A, B, C, and D) of the SHIAP H18 gene, generously supplied by M. Ono (26). Inserts were purified from pBR322 vector by agarose gel electrophoresis after restriction endonuclease cleavage. All CHIAP cDNA clones described here showed hybridization through two rounds of plaque hybridization screening and DNA blot analysis of λ DNA prepared from plaques after screening.

DNA sequencing and sequence analysis. Inserts from λ clones were subcloned into pUC vectors and the M13 vectors, mp18 or mp19, for sequencing. Some subclones were prepared in pUC219, a pUC19 derivative containing the intergenic region of M13. This allowed preparation of single-stranded template for sequencing after infection with the helper bacteriophage M13K07 (kindly supplied by J. Viera). DNA sequencing was performed, using the dideoxy-chain termination method on single-stranded template (31). M13 universal primer was used to generate initial sequence, and subsequent sequence was derived by using synthetic oligonucleotide extension primers.

Sequence comparisons and alignments were done by computer analysis, using the algorithm of Needleman and Wunsch (25) as described by Fitch and Smith (6), with enhancements suggested by Gotoh (7). For polypeptide sequence alignment, the mutation data matrix (PAM250) described by Dayhoff et al. (4) was applied, using deletion penalties described by Lipman and Pearson (15).

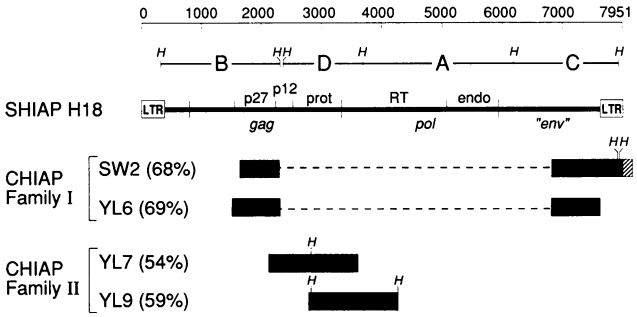

FIG. 1. Schematic representation of CHIAP cDNA clones with respect to the SHIAP H18 genome. The scale at the top represents nucleotides of the SHIAP H18 sequence (26). Below it are the four *Hin*dIII fragments of SHIAP H18 used as probes for plaque screening and blot hybridizations. Solid bars represent regions of nucleotide homology of CHIAP cDNA clones to the SHIAP H18 genome. Dashed lines represent intervening sequences not present in the CHIAP cDNA clones. The hatched region represents the presumed flanking cellular sequences present in the CHIAP.SW2 clone. *Hin*dIII sites are indicated by *H*. Percentages refer to overall nucleotide homology of the CHIAP sequences to the SHIAP H18 sequence over the regions indicated. prot, Protease; RT, reverse transcriptase; endo, endonuclease.

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed by using the GeneAmp system (Taq DNA polymerase; Perkin-Elmer/Cetus) and an automated thermal cycler. High-molecular-weight cellular DNA was initially denatured for 12 min at 94°C followed by 30 cycles of primer annealing and elongation. Annealing was at 50°C for 1.5 min and elongation was at 72°C for 2.5 min. Denaturation between cycles was at 94°C for 1 min. A final elongation step of 12 min was used to ensure that all amplified sequences were extended to completion. Cellular DNA (100 ng) or 100 pg of plasmid DNA was used for each reaction. Synthetic oligonucleotides (20 or 22 nucleotides) were used as the primers at final concentrations of 0.3 μM. Double-stranded products of the PCR were fractionated on 5% polyacrylamide gels and visualized under UV light after being stained with ethidium bromide.

DNA blot analysis. High-molecular-weight DNA was prepared from cells by standard methods (20) and digested with *Hin*dIII restriction endonuclease (New England BioLabs, Inc.) under conditions recommended by the supplier. Complete digestion of DNA was monitored by evaluation of ethidium bromide-stained gel patterns of samples removed from the digestion mixture at 2-h intervals. When the gel pattern failed to shift to a lower-molecular-weight range after two subsequent samplings of DNA, enzyme equivalent to that originally used was added and the digestion was allowed to proceed for an equivalent amount of time. The DNA was judged to be digested to completion if no further evidence of a change in molecular weight range was observed.

DNA was fractionated by electrophoresis on agarose gels, denatured, neutralized, transferred to membranes (GeneScreen Plus; Dupont, NEN Research Products) by capillary blotting in 10× SSC, and hybridized with probes labeled with [α-^{32}P]dCTP by nick translation. Hybridization was performed overnight, using both high- and low-stringency conditions. High-stringency hybridization was performed in 50% formamide–0.5 M NaCl–0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.2)–5 mM

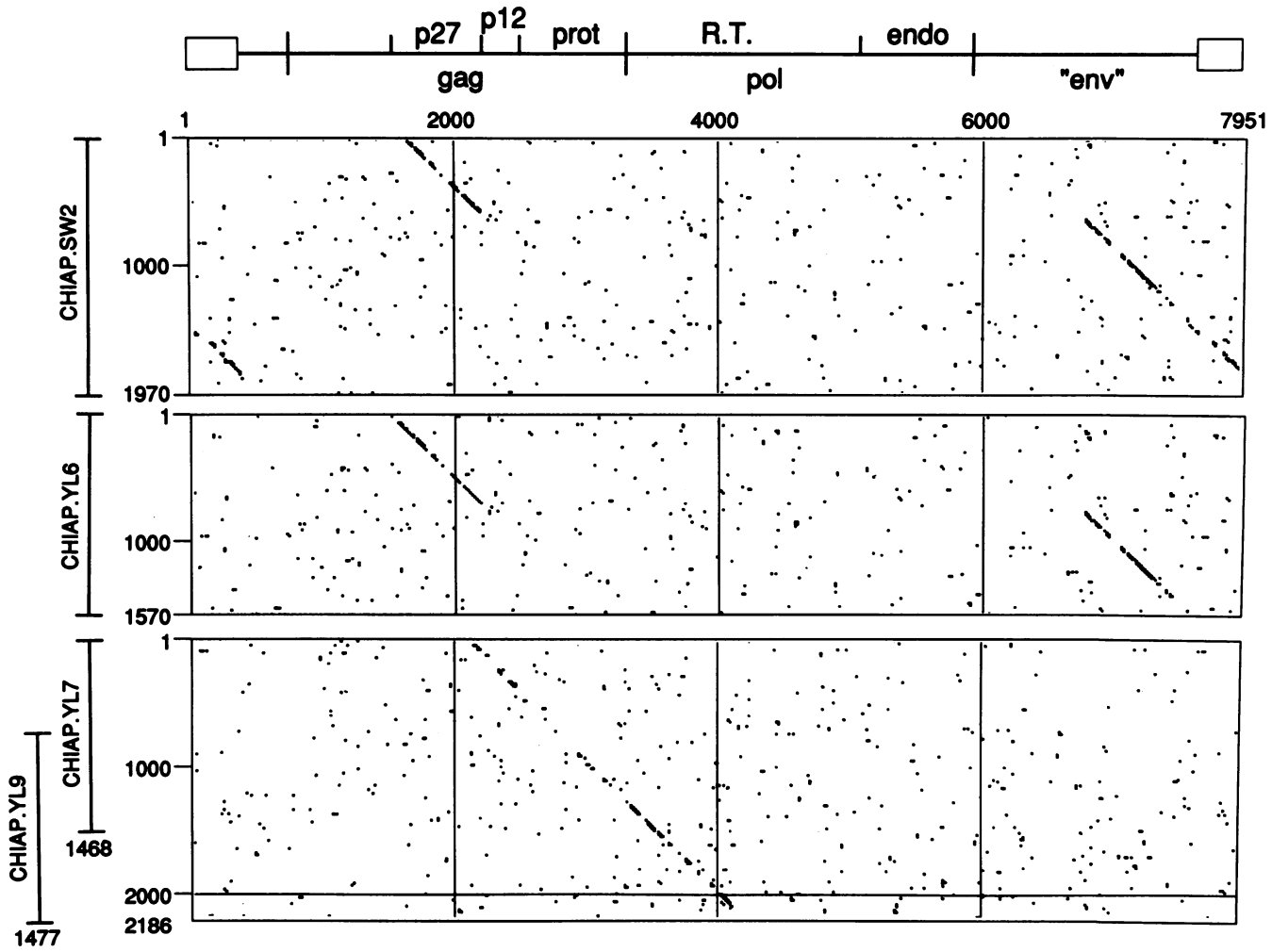

FIG. 2. Homology of CHIAP cDNA sequences to SHIAP sequence. The sequence of the SHIAP H18 genome (26) is plotted on the horizontal axis, and sequences of the CHIAP cDNA clones are plotted on the vertical axes. Each dot represents homology of 75% over a stretch of 16 nucleotides. Diagonal lines represent regions of significant homology. prot, Protease; R.T., reverse transcriptase; endo, endonuclease.

EDTA–0.1% sodium pyrophosphate–5× Denhardt solution–100 μg of denatured salmon sperm DNA per ml at 42°C with final rinses in 0.1× SSC–0.1% sodium dodecyl sulfate at 42°C. Low-stringency hybridization was performed identically except that the formamide concentration used for hybridizations was 25% and final rinses were in 2× SSC–0.1% sodium dodecyl sulfate. Filters were pretreated for 4 to 6 h before hybridization in the appropriate hybridization buffer without probe.

RNA blot analysis. Unless otherwise stated, cytoplasmic RNA isolated from cells by phenol-chloroform extraction of 0.5% Nonidet P-40 supernatants was used for all experiments. In some cases, poly(A)$^+$ RNA was prepared by fractionation over oligo(dT) cellulose. After ethanol precipitation, RNA samples were electrophoresed in denaturing formaldehyde agarose gels and blotted onto membranes essentially as described by Maniatis et al. (20). GeneScreen Plus membranes (Dupont, NEN Research Products) were used, and the transfer buffer was 12 mM Tris (pH 7.8)–6 mM sodium acetate–0.3 mM EDTA. Hybridization and rinses were as described above for DNA blots.

RESULTS

Identification and characterization of cDNA clones related to IAP genes. Several clones containing IAP-related sequences were isolated from a CHO cell cDNA library. Cloned *Hin*dIII fragments of the SHIAP H18 genome were used as hybridization probes under low-stringency conditions to identify recombinant bacteriophage with homologous inserts. The orientation and approximate sizes of the fragments used as probes are depicted in Fig. 1. The high frequency of detection of IAP-related clones in the λ library (as high as 1 in 3,000 plaques for some probes) indicated that IAP-related poly(A)$^+$ RNA is relatively abundant in the cytoplasm of CHO cells.

Inserts from several of the clones which hybridized to the SHIAP probes through two rounds of hybridization were subcloned into plasmid or M13 vectors for further characterization. Inserts from four isolates were sequenced in their entirety. All four clones exhibited sequence similarity to the SHIAP genome. However, the clones could be segregated into two distinct families on the basis of the extent of

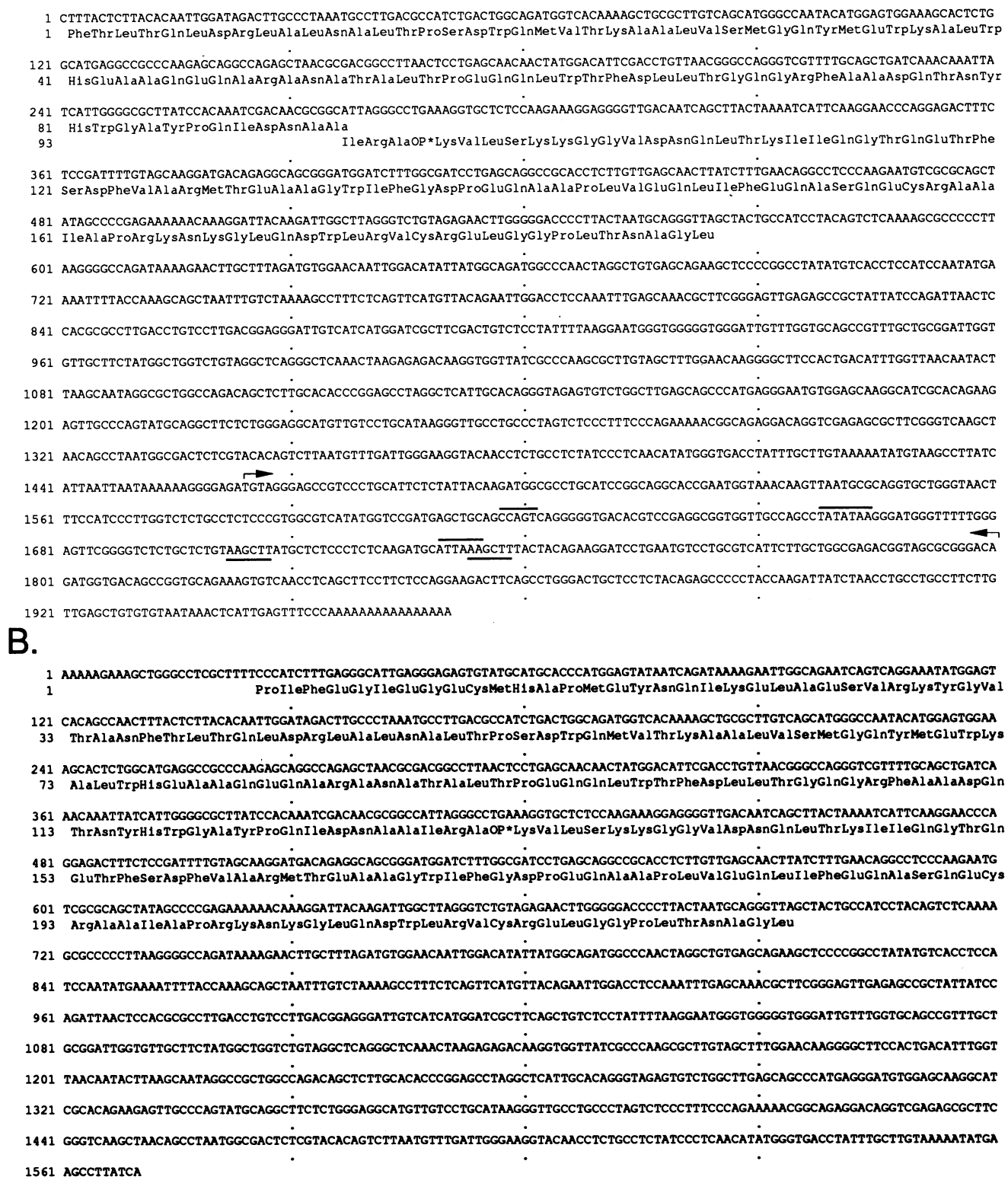

FIG. 3. Nucleotide sequence of CHIAP cDNA clones CHIAP.SW2 (A), CHIAP.YL6 (B), and CHIAP.YL7/9 (C). Sequences of strands colinear with the coding strand of the SHIAP H18 genome (26) are shown. Potential translation products of the deduced p27 gene (A and B) and of the deduced protease gene (C) are shown. The arrows above the nucleotide sequences in panel A represent the inferred limits of an LTR, and solid lines above the sequences represent potential regulatory signals as discussed in the text. *Hin*dIII sites are underlined. Sequences at the 3′ end of YL7 and the 5′ end of YL9 are identical over a stretch of 759 nucleotides. Therefore, their combined sequence, without duplication of the overlap (nucleotides 710 to 1468), is shown (C).

C.

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   1 GCAATAACTCCATATAAGGGTAAAGGCCTTGAAGTCTGGATGAAAGTCTGTAGGGAGTTAGGGGGTCCGCTGACTAATGCTGGACTAGCAGCTGCTGTGTTGCAATTAACTAAGAAAGGT
 121 GGAGGTTCAGGAGCTTGCTTTAAATGCGGCAAGCAAGGGCATTTGAAAAAGCAATGCCCCGAGGGAGGAAACACTAAAGTCAATAAACTTTGCTCCGCGCCCTAAGCAACCTGGCTTATG
 241 TCCTAGATGTAGAAAAGGAAATCATTGGGCTAAGGATTGTAGATCAGTAAAAGACATCAGTGGACAGCCTCTTGTTCAGGGGTATGGAGGAGCCCGTTCAAAAAACGGACGACGGGGCCC
 361 ACGACCCCAGGGCCCACAAATATATGGGGCCATGGAGGATCAGAACCAGGAGCAGAGTCCCGAAACCTGGCCCTCTCTTCGTCATCCGAGGGACCGAGGAGAGCCACTACAGGCTCCGCG
 481 GGGCTGGACTTACGCTCCACCACCAGACTCGTATTAACTCCCAGAATGGGGGTCCAGCTTGTTGACACCGATTTTAAGGGACCCCTTGAGCCTGGCACAGTAGGTTTGCTTATAGGAAGA
   1                                                                                                  PheLysGlyProLeuGluProGlyThrValGlyLeuLeuIleGlyArg
 601 TCATCTGCAGCATTGAAAGGTTTACGAGTACATCCTGGAGTTATAGATCCTGATTACATGGGTGTAGTAAAGATCATGGTAGAATCTCCTAGAGGGATTACGGCCATTTCTCCTGGAGAC
  17 SerSerAlaAlaLeuLysGlyLeuArgValHisProGlyValIleAspProAspTyrMetGlyValValLysIleMetValGluSerProArgGlyIleThrAlaIleSerProGlyAsp
 721 AGGATAGCACAGTTACTGCTTTTGCCAAGCTTGCATGACAAGTTTCCAGCACAAGCCAGAGAGAGAGGAGAGGGAAACTTTGGCTCCACTGGATCAAACTTAACTTTCCTAGCTTTAGAC
  57 ArgIleAlaGlnLeuLeuLeuLeuProSerLeuHisAspLysPheProAlaGlnAlaArgGluArgGlyGluGlyAsnPheGlySerThrGlySerAsnLeuThrPheLeuAlaLeuAsp
 841 CTTGATCAACGTCCAACCCTTGAGTTAATAGTGAATGGTAAGAAAATCTTAGGCTTACTAGATTCTGGAGCTGATAAGAGCATCATAGCCACTAAAGATTGGCCCTCTGGCTGGCCTATA
  97 LeuAspGlnArgProThrLeuGluLeuIleValAsnGlyLysLysIleLeuGlyLeuLeuAspSerGlyAlaAspLysSerIleIleAlaThrLysAspTrpProSerGlyTrpProIle
 961 CAGGTTTCTTCTCAAAGTTTACAAGGTTTAGGCTATGCTAAGGCTCCTGATATGAGTGCTAGACAATTGCCTTGGAAAGATCAGGAAGGGCATTCAGGGACCATGCAACCTTATGTGTTA
 137 GlnValSerSerGlnSerLeuGlnGlyLeuGlyTyrAlaLysAlaProAspMetSerAlaArgGlnLeuProTrpLysAspGlnGluGlyHisSerGlyThrMetGlnProTyrValLeu
1081 GACTTACCAATTTCATTATGGGGAAGAGATTTGTTAAAGGATATGGGTTTTAAACTCACAAATGAATACTCAGAAACATCTCAAGGTATCATGAAACGAATGGGATACAGTCCCAGGCCA
 177 AspLeuProIleSerLeuTrpGlyArgAspLeuLeuLysAspMetGlyPheLysLeuThrAsnGluTyrSerGluThrSerGlnGlyIleMetLysArgMetGlyTyrSerProArgPro
1201 GGCCTCGGGAAACATCTGCAGGGTCGTACCAGTCCTATTAATTCCACAATTGAGACCAAAGAATCTAGGTCTGGGTTTTTCCTAGGGCCACTGAGGAGGTATTCCTATTACCTGGAAAAC
 217 GlyLeuGlyLysHisLeuGlnGlyArgThrSerProIleAsnSerThrIleGluThrLysGluSerArgSerGlyPhePhe
1321 AGAGGAGCCGGTATGGGTTCCTCAGTGGCCACTTTCCTCTGAGAAACTGGAAGCTGCTAAGACTCTAGTGCGGGAGCAGCTGGATCTGGGGCATATAAAATCCTCTGTATCTCCATGGAA
1441 TACTCCTATTTTTGTCATTAAGAAAAAATCTGGTAAATGGAGACTGCTTCACGATCTTAGAGCTATTAATCAACAGATGCAAATTATGGGCCCTGTACAACGTGGTCTTCCACTTTTAAC
1561 TTCTTTACCTGCATCATGGCCTATCATCTCTATAGATATTAAAGATTGCTTCTTTTCCATACCTTTGTGTGCCAAGGATTCAGGGCGTTTTGCGTTTACGCTGCCCTCTTGTAATCATGA
1681 ACAACCTGATTTAAGGTATGAATGGGATAGTGTTGGCCACAGGGGATGGCCAATAGTCCTACTATGTGTCAGTTGTTTGTAGCAGAAGCAATTGCTCCTTTTGAGAGTGGACTTTCCCAA
1801 AGATTAGATGTGTTCATTATATGGATGATATTTTATTGGCTGCCAAAGATGATAAAACGCTTAATAAGGCATATACAAAATTGGTAAAATTGCTTGAGATGCATAATTTAGTCATAGCCT
1921 CAGAAAAGGTACAAAAGGACACTGTTGTTAACTATCTAGGGGCTAAGATTCTCCCTCATACAATTATTCCACAAAAGATAGAGATTAGAAAAGATAATTTAAAAACTCTTAATGATTTTC
2041 AAAAGTTGTTGGGAGATATAAATTGGATAAGATGTTATTTAAAATTACCAAATTATGAGTTGAAGCCATTGTATAATATTCTCAATGGTGATTCAGCATTAGATTCACCTAGGCAGTTAA
2161 CTGCTGAAGCCAGAGAAGCTTTAAAG
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FIG. 3—*Continued.*

homology to the SHIAP genome and the presence of apparent internal deletions in two of the clones. The inferred relationship of CHIAP clones to SHIAP sequences is represented diagrammatically in Fig. 1. Homology plots comparing cloned CHIAP sequences with the published sequence of a cloned full-length SHIAP gene (26) are shown in Fig. 2. Actual nucleotide sequences of the four CHIAP clones are presented in Fig. 3.

Two clones, CHIAP.SW2 (1,970 base pairs [bp]) and CHIAP.YL6 (1,570 bp), represent family I sequences. CHIAP.SW2 was originally identified by virtue of its ability to hybridize to a mixture of *Hin*dIII fragments representing the 5′ end of the SHIAP H18 genome (fragments B and D) (Fig. 1) during a primary round of plaque screening. Sequence similarity was confirmed during a second round of screening, using a probe representing the 3′ end of the SHIAP H18 genome (*Hin*dIII fragment C). CHIAP.YL6 was isolated from the cDNA library after two rounds of plaque screening, using *Hin*dIII fragment B of the SHIAP H18 genome (5′ end) as probe. CHIAP.SW2 and CHIAP.YL6 sequences are 68 and 69% homologous, respectively, to the SHIAP H18 sequence when optimally aligned (Fig. 1 and 2). Homology plots with parameters identical to those of the plots shown in Fig. 2 revealed no significant homology of either of these clones to evolutionarily related B-type (MMTV [23]) or D-type (simian retrovirus type 1 [SRV-1] [29]) retroviruses (not shown). CHIAP.SW2 and CHIAP.YL9 differ from each other at only 6 bases in an overlap of 1,441 bases. Both of these independently isolated clones exhibit identical large sequence discontinuities with respect to the SHIAP H18 sequence. In an optimal alignment of homologies, the discontinuity extends from nucleotides 2265 to 6775 of the SHIAP H18 sequence.

A comparison of CHIAP family I sequences to those of the SHIAP H18 and mouse MIA14 IAP genomes (22, 26) revealed the presence of a region in the CHIAP.SW2 clone likely to represent an intact long terminal repeat (LTR). On the basis of an optimal alignment of CHIAP.SW2 and SHIAP H18 sequences, the CHIAP.SW2 LTR extends from nucleotide 1464 through nucleotide 1800 of the CHIAP.SW2 sequence. The size of the CHIAP.SW2 LTR (337 bp) is precisely that reported for the murine IAP MIA14 LTR and is slightly smaller than that reported for the SHIAP H18 LTR (376 bp). Homologies of the CHIAP.SW2 LTR sequence with the corresponding regions in the SHIAP H18 and the murine IAP MIA14 were 69 and 66%, respectively. Several putative regulatory sequences were conserved within the CHIAP.SW2 LTR and are indicated in Fig. 3A. The TATA box (nucleotides 1658 to 1664) and polyadenylation signals (nucleotides 1729 to 1734) have been precisely conserved when compared with the SHIAP H18 sequence. The CAAT box (nucleotides 1617 to 1621) has been conserved, except

for a single-base-pair substitution (CCAAT→CCAGT). The CHIAP.SW2 LTR contains a single core enhancer sequence (nucleotides 1524 to 1532) characteristic of the murine IAP MIA14 LTR (22) rather than multiple direct repeats as observed for the SHIAP H18 LTR (26). A polypurine tract reported just upstream of the 3′ LTRs of both murine and SHIAP genes was also observed upstream of the CHIAP.SW2 LTR (nucleotides 1452 to 1463).

Clones CHIAP.YL7 (1,468 bp) and CHIAP.YL9 (1,477 bp) represent family II sequences. Both were isolated from the CHO cell cDNA library after two rounds of plaque screening, using *Hin*dIII fragment D of the SHIAP H18 genome as the probe. This fragment represents the *gag-pol* junction region of the SHIAP H18 genome (Fig. 1). Inserts from the CHIAP.YL7 and CHIAP.YL9 clones exhibit less similarity to the SHIAP H18 genome than do inserts from family I clones (54% for CHIAP.YL7 and 59% for CHIAP.YL9) but are identical to each other over a 759-bp region of overlapping sequence. The combined sequence of these overlapping clones (designated CHIAP.YL7/9) is 58% homologous to the SHIAP H18 genome. The CHIAP.YL7/9 sequence is colinear with the SHIAP H18 sequence and does not exhibit any significant discontinuities relative to the SHIAP H18 sequence, as observed for family I clones. Nucleotide homology plots similar to that in Fig. 2 revealed that homologies of family II sequences with MMTV and SRV-1 genomes are comparable to the homology of family II sequences with the SHIAP genome (not shown). Optimal alignments with MMTV and SRV-1 genomic sequences showed 53 and 56% nucleotide identity, respectively.

For the most part, family II sequences are homologous to regions of the IAP genome different from those to which family I sequences are homologous. However, a small region of overlap was observed between the 5′ sequence of CHIAP.YL7 and internal sequences of the CHIAP.SW2 and CHIAP.YL6 clones. This overlap occurs over 147 bp just 5′ of the discontinuity observed in family I sequences. The homology between family I and family II sequences in this region is just 63%, providing evidence that the two families are related but not identical.

Detection of CHIAP family I deletion in genomic DNA of CHO cells. PCR was used to determine whether the sequence discontinuities in family I clones represented deletions of genomic DNA or splicing of mRNA used as template for preparation of the cDNA clones. Oligonucleotides complementary to sequences on either side of the discontinuity in the family I clones and with converging polarities were synthesized and used as the primers to amplify intervening sequences present in the genomic DNA of CHO cells. Figure 4 shows the results of an experiment using one set of primers. Similar results were obtained with a second set of primers (not shown). PCR of DNA from CHO-DUX and CHO-C2B cells resulted in the amplification of a predominant DNA fragment which comigrated with the amplification product of CHIAP.SW2 plasmid DNA and corresponded in length to that predicted from the DNA sequence. Therefore, sequences exhibiting a discontinuity of the same size and in the same location as that observed in family I cDNA clones exist in the genomic DNA of CHO cells. This information as well as the absence of splice site consensus sequences (24) in the region of the deletion for either murine or SHIAP sequences suggests that the sequence discontinuity in our cDNA clones reflects the transcription of a deleted gene rather than the splicing of RNA from an intact transcript.

Family I and family II sequences in genomic DNA of CHO cells. Blot analysis of CHO cell DNA with cloned CHIAP cDNA probes allowed estimation of the number of copies of CHIAP sequences present in the genome of CHO cells (Fig. 5). For the characterization of family I sequences, a modified insert from the CHIAP.SW2 clone was used as the probe. Presumed cellular sequences were removed from one end of this clone, using the *Hin*dIII site at nucleotide position 1702. Hybridization of this probe to *Hin*dIII-digested DNA from CHO-DUX or CHO-C2B cells revealed the presence of five distinct bands of comparable intensity representing DNA fragments ranging in size from 1.4 to 2.6 kbp (Fig. 5A). The intensity of hybridization of each of these prominent bands was approximately the same as that of plasmid reconstruction controls representing 100 copies of CHIAP.SW2 sequence per haploid CHO cell genome. The total number of hybridizing sequences present in CHO cell DNA was estimated to be 200 to 500 copies per haploid genome. Hybridization of the CHIAP.SW2 probe to DNA of mouse myeloma or human diploid MRC5 cells was not detected by using high-stringency hybridization conditions (Fig. 5A), but hybridization to mouse myeloma cell DNA was detected by using low-stringency conditions (not shown).

For the characterization of family II sequences, an insert from the CHIAP.YL9 clone was used to probe blots of *Hin*dIII-digested CHO cell DNA. By using this probe, a pattern of hybridization different from that for the family I probe was observed (Fig. 5B). The most intense hybridization was to a fragment which comigrated with a 1,429-bp fragment resulting from *Hin*dIII cleavage of the CHIAP.YL9 plasmid. The intensity of hybridization to this band was

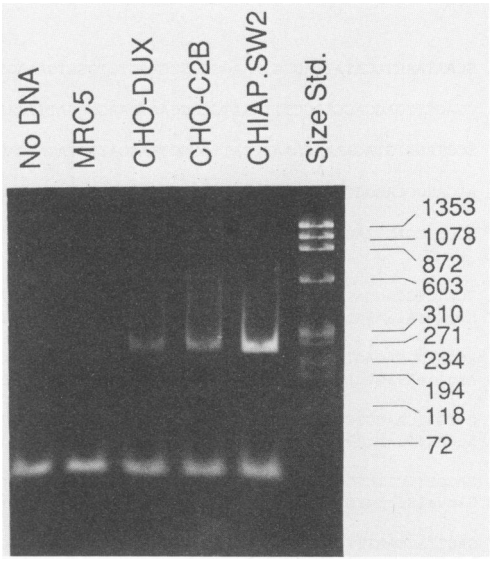

FIG. 4. PCR analysis of genomic DNA from CHO cells. PCR was used to amplify CHIAP family I sequences present in the DNA of indicated cell lines (100 ng) or CHIAP.SW2 plasmid (100 pg). Primers on either side of the sequence discontinuity observed in CHIAP family I cDNAs relative to murine and SHIAP genes (nucleotides 498 to 517 and the complement of nucleotides 755 to 734 of the CHIAP.SW2 sequence) (Fig. 3A and 7A) were used to determine whether the same sequence discontinuity is present in the genomic DNA of CHO cells. A photograph of an ethidium bromide-stained polyacrylamide gel of PCR amplification products is shown. The slower-migrating band present in the CHO cell and plasmid lanes is the predicted size for the amplified product representing CHIAP family I sequences (257 bp). The faster-migrating band observed in all the lanes represents amplified primer dimers. Size standards are *Hae*III restriction fragments of ϕX174 DNA.

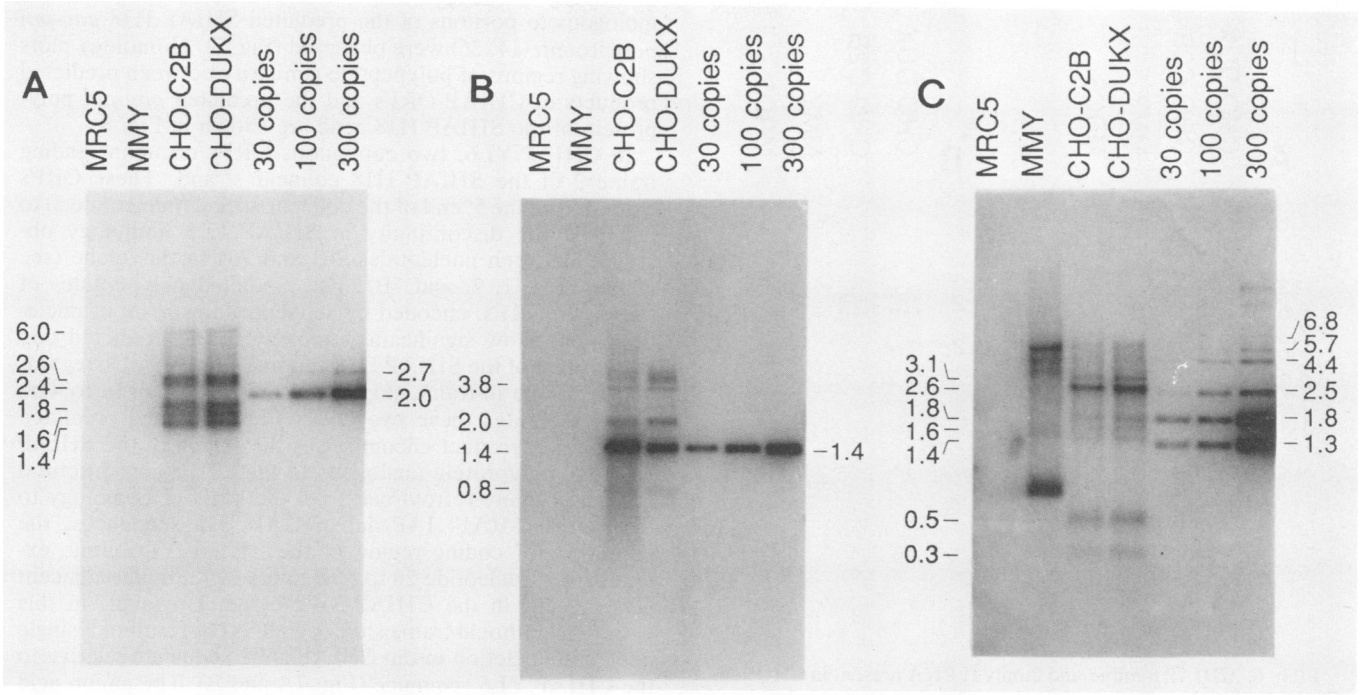

FIG. 5. DNA blot analysis of CHIAP sequences in CHO cells. *Hin*dIII-digested DNA (2 μg per lane) from human diploid fibroblasts (MRC5), mouse myeloma cells (MMY), and CHO cell lines (C2B and DUX) was blotted onto membranes and hybridized with CHIAP or SHIAP probes. Numbers to the left of the panels indicate sizes (in kilobase pairs) of hybridizing fragments of CHO cell DNA deduced from the migration of λ *Hin*dIII fragments in the same gel. Numbers to the right of the panels indicate known sizes (in kilobase pairs) of fragments in copy number control lanes. Copy number controls are restricted plasmids loaded onto the gel such that the amount of insert is equivalent to the indicated number of copies per CHO cell haploid genome. (A) Hybridization with the CHIAP.SW2 probe representing family I sequences. Since only one *Hin*dIII site was identified in cloned family I sequences, a full-length *Eco*RI insert from the CHIAP.SW2 plasmid was used as the copy number control. (B) Hybridization with the CHIAP.YL9 probe representing family II sequences. Nucleotide sequence information showed the presence of an internal 1,429-bp fragment within the CHIAP.YL9 sequence. Therefore, the copy number in this case was *Hin*dIII-digested CHIAP.YL9 plasmid. (C) Hybridization with the SHIAP H18 probe. An equimolar mixture of four cloned *Hin*dIII fragments (fragments A, B, C, and D) representing the entire SHIAP H18 genome (Fig. 1) was used as a probe. The copy number control was the *Hin*dIII-digested DNA of three of the four plasmids whose inserts were used as probe (fragments A, C, and D). Hybridizations in panels A and B were performed under high-stringency conditions, and hybridization in panel C was performed under low-stringency conditions (see Materials and Methods).

intermediate to that observed for *Hin*dIII-digested CHIAP.YL9 plasmid control at concentrations equivalent to 100 or 300 copies per haploid CHO cell genome. Less intense hybridization to other discrete bands was also observed. Hybridization of the YL9 probe to human MRC5 cell DNA or to mouse myeloma cell DNA was not detected by using high- or low-stringency hybridization conditions.

Blots of *Hin*dIII-digested CHO cell DNA were also probed with an equimolar mixture of the four *Hin*dIII fragments (A, B, C, and D) representing the entire SHIAP H18 genome (Fig. 1). Hybridization of the blot shown in Fig. 5C was performed by using low-stringency conditions so that partially homologous sequences could be detected. Both CHO cell lines exhibited one prominent band of hybridization (2.6 kbp) as well as several other less prominent bands. The major band and several minor bands correlated in size with those detected by using homologous CHIAP probes. A comparison of hybridizing *Hin*dIII fragments from CHO cell DNA with *Hin*dIII fragments of the SHIAP H18 genome revealed that *Hin*dIII sites from SHIAP sequences have not been appreciably conserved in CHIAP sequences. Significant hybridization of the SHIAP probe to the DNA of the mouse myeloma cells was also observed under low-stringency conditions, consistent with previously reported results demonstrating the presence of abundant murine IAP sequences in the DNA of several murine myeloma cell lines (14). No detectable hybridization of the SHIAP H18 probe to the DNA of MRC5 cells was observed by using either low- or high-stringency conditions.

CHIAP RNA transcripts in CHO cells. RNA blot analysis was used to evaluate transcription products of CHIAP sequences in CHO cells. When the modified CHIAP.SW2 insert described above was used as a probe for family I-encoded RNA, qualitatively similar patterns of hybridization were observed for the parental CHO-DUX cells and the recombinant CHO-C2B cells (Fig. 6A). A predominant 2.9-kilobase (kb) mRNA species of comparable intensity was observed in each, but the intensity of other bands on the blot differed between the two cell lines. Blots of poly(A)$^+$ RNA revealed similar patterns (data not shown). Quantitative slot blot analysis of poly(A)$^+$ RNA from CHO-DUX cells revealed that RNA homologous to the CHIAP.SW2 probe composed approximately 0.01% of the cytoplasmic poly(A)$^+$ RNA (not shown).

Hybridization of the CHIAP.SW2 probe to RNA from mouse myeloma cells was also observed. The two bands observed (7.6 and 5.5 kb) probably represent the predominant IAP mRNA species previously reported in MOPC-21 mouse myeloma cells as 7.2 and 5.4 kb in size (13). Hybridization of the CHIAP.SW2 probe to RNA but not to DNA from mouse myeloma cells under high-stringency conditions (Fig. 5A and 6A) probably reflects the greater stability of

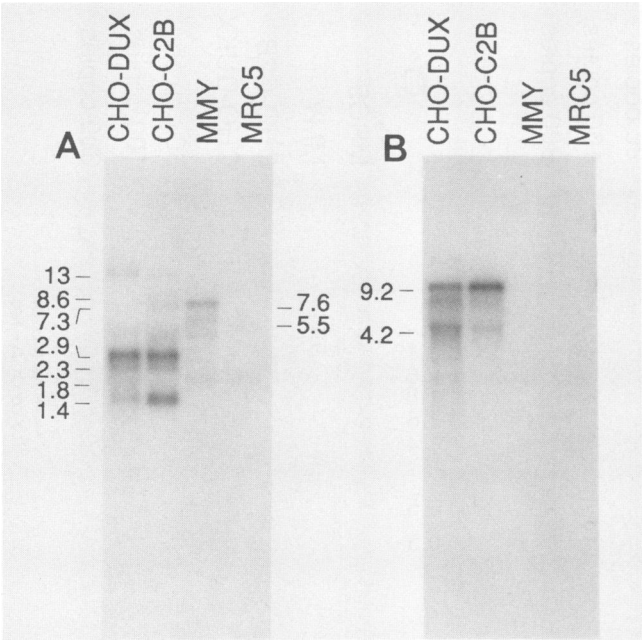

FIG. 6. CHIAP family I and family II RNA present in CHO cells. Cytoplasmic RNA from indicated cell lines (4.5 μg per lane) was electrophoresed on formaldehyde agarose gels, blotted onto membranes, and hybridized with probes specific for family I (CHIAP.SW2) (A) or family II (CHIAP.YL9) (B) sequences, using high-stringency conditions. Numbers to the left of the panels indicate sizes (in kilobases) of major RNA species in CHO cells as deduced from the migration of unlabeled RNA size markers. Numbers to the right of panel A represent deduced sizes (in kilobases) of RNA species in mouse myeloma cells (MMY) hybridizing to the CHIAP.SW2 probe.

nonidentical RNA-DNA hybrids compared with that of DNA-DNA hybrids under the same conditions. The RNA transcripts detected (Fig. 6A) are likely to be products of DNA sequences detected in mouse myeloma cells by using the same probe under lower-stringency hybridization conditions (see above). No specific hybridization of the CHIAP.SW2 probe to RNA of human MRC5 cells was observed.

The CHIAP.YL9 insert was used as a probe to detect RNA homologous to family II CHIAP sequences on blots. Hybridization with this probe revealed a pattern different from that observed with the family I probe (Fig. 6B). Only two RNA species were detected in both CHO-DUX and CHO-C2B cells by using the CHIAP.YL9 probe (4.2 and 9.2 kb). The 9.2-kb transcript is of an appropriate size for a full-length retrovirus transcript (40) and is comparable to the reported and predicted sizes of full-length transcripts from murine and SHIAP genes (7.2 and 8.1 kb, respectively) (13, 22, 26). This band was not detected with the family I probe even under low-stringency conditions. Quantitative slot blot analysis of CHO-DUX poly(A)$^+$ cytoplasmic RNA indicated that mRNA homologous to the CHIAP.YL9 probe represented approximately 0.3% of cytoplasmic poly(A)$^+$ mRNA in CHO cells (data not shown). No hybridization of the CHIAP.YL9 probe to the RNA of either mouse myeloma cells or human diploid MRC5 cells was observed.

Polypeptide encoding potential of CHIAP clones. When family I and family II DNA sequences were analyzed for polypeptide encoding potential, no intact *gag*, *pol*, or *env* open reading frames (ORFs) were present. However, several incomplete ORFs with predicted polypeptide products homologous to portions of the predicted SHIAP H18 *gag-pol* polyprotein (14, 26) were observed (Fig. 7). Homology plots showing regions of polypeptide similarity between predicted products of CHIAP ORFs and the predicted *gag-pol* polyprotein of the SHIAP H18 gene are shown in Fig. 8.

In CHIAP.YL6, two contiguous ORFs occur in reading frame 2 of the SHIAP H18 colinear strand. These ORFs extend from the 5′ end of the colinear strand (nucleotide 2) to just past the discontinuity in SHIAP H18 homology observed between nucleotides 763 and 764 in this clone (see above) (Fig. 1, 2, and 7B). The predicted polypeptides of these two ORFs, encoded by sequences upstream of nucleotide 764, show significant homology to the predicted *gag* polyprotein of the SHIAP H18 genome (Fig. 8A). Disregarding the single in-frame stop codon (nucleotides 416 to 418) which separates these two ORFs, the combined predicted polypeptide product encompasses the region of the SHIAP H18 *gag* polyprotein analogous to the p27 major structural protein of many retroviruses. On the basis of homology to the murine MIA14 IAP and SHIAP H18 sequences, the potential p27 coding region of the CHIAP.YL6 clone extends from nucleotide 26 to nucleotide 694. Similar adjacent ORFs occur in the CHIAP.SW2 clone. However, in this clone an additional frame shift occurs as the result of a single nucleotide deletion in the CHIAP.SW2 sequence relative to the CHIAP.YL6 sequence (Fig. 7A and B). The amino acid sequence of the presumptive p27 polypeptide encoded by the two adjacent ORFs in CHIAP.YL6 was compared with the amino acid sequences of p27-related polypeptides from several related retroviruses (Table 1). The predicted p27 products of murine and SHIAP genes exhibited the greatest homology with the CHIAP.YL6 p27 sequence, but homologies to p27 amino acid sequences of MMTV and SRV-1 were also noted.

Because the family II clones, CHIAP.YL7 and CHIAP.YL9, exhibited nucleotide sequence identity in overlapping regions, an analysis of potential family II ORFs was performed, using the combined nucleotide sequence designated CHIAP.YL7/9. Multiple incomplete ORFs with predicted polypeptide products homologous to the predicted SHIAP H18 *gag-pol* polyprotein were observed on the SHIAP H18 colinear strand (Fig. 7C). However, the regions of homology were often in different reading frames as a result of multiple small insertions and deletions relative to the SHIAP H18 coding sequence. The regions of homology between predicted polypeptides of CHIAP ORFs and the SHIAP H18 *gag-pol* polyprotein are adjacent and nonoverlapping even though they often occur in different reading frames. This is most clearly evident in polypeptide homology

TABLE 1. Amino acid homology of putative polypeptide products of CHIAP clones

Origin of sequence used for comparison (reference)	Homology to p27 of CHIAP family I		Homology to protease of CHIAP family II	
	Score[a]	% Identity	Score[a]	% Identity
Murine IAP (14, 22)	962	77	539	44
SHIAP (14, 26)	833	70	630	49
SRV-1, D-type (14, 29)	407	38	516	42
MMTV, B-type (23)	270	29	506	47
RSV,[b] avian C-type (32)	140	25	144	30

[a] Modified Dayhoff mutation data matrix (PAM250) score evaluates the significance of individual amino acid matches and substitutions (see Materials and Methods).

[b] RSV, Rous sarcoma virus.

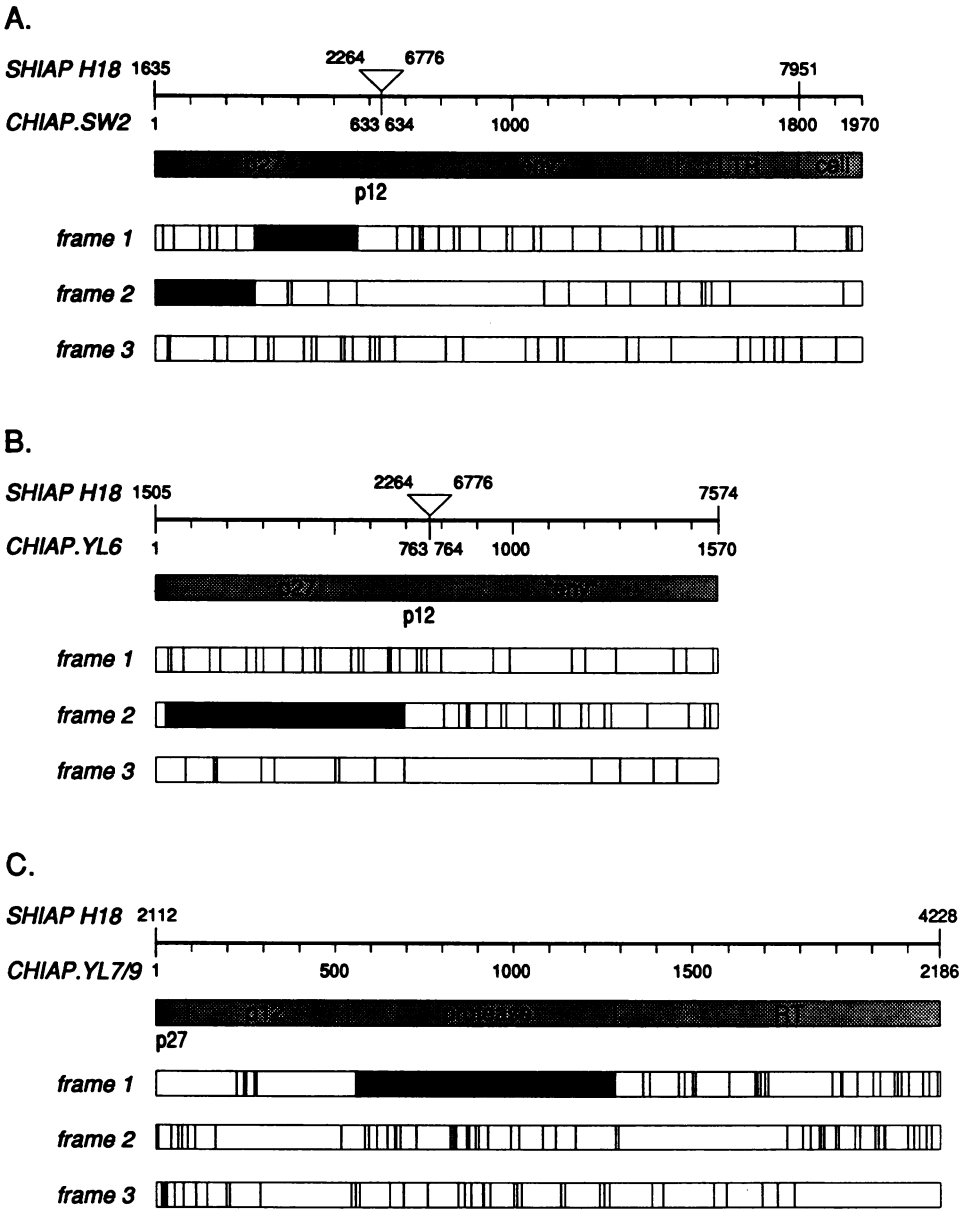

FIG. 7. ORFs of CHIAP cDNA clones CHIAP.SW2 (A), CHIAP.YL6 (B), and CHIAP.YL7/9 (C). Stop codons in each reading frame of the CHIAP sequences are indicated by vertical lines. Only reading frames of the strand colinear with the coding strand of the SHIAP H18 genome are shown. Significant ORFs on the opposite strand were not observed. Solid bars indicate regions with potential coding capacity for p27 and protease-related polypeptides. Stippled bars indicate corresponding regions of the SHIAP H18 genome as deduced from nucleotide homologies. Nucleotide coordinates indicating regions of homology and major deletions relative to the SHIAP H18 genome are shown on the top line of each panel. RT, Reverse transcriptase.

plots (Fig. 8B). These adjacent and nonoverlapping regions of polypeptide homology reflect the colinearity of the CHIAP.YL7/9 and the SHIAP H18 nucleotide sequences.

Homology of the CHIAP.YL7/9 predicted polypeptide products to the SHIAP H18 *gag-pol* polyprotein extends from near the C terminus of the p27 domain to the middle of the reverse transcriptase domain. However, the multiple frame shifts which occur make interpretation of the significance of these predicted polypeptide products difficult. Only one ORF had potential encoding capacity sufficient for an intact, recognizable polypeptide domain. This ORF occurred in reading frame 1 of the CHIAP.YL7/9 sequence and exhibited coding potential sufficient for an intact protease polypeptide. The presumptive protease polypeptide encoded by the CHIAP.YL7/9 sequence showed amino acid sequence homology to the presumptive protease polypeptides encoded by the SHIAP H18, murine IAP MIA14, MMTV, and SRV-1 genomes (Table 1).

DISCUSSION

We have isolated four cDNA clones from a CHO cell library which represent transcription products of endogenous retroviruslike elements in CHO cell DNA. The clones were selected by their abilities to hybridize under low-stringency conditions to probes representing a SHIAP ge-

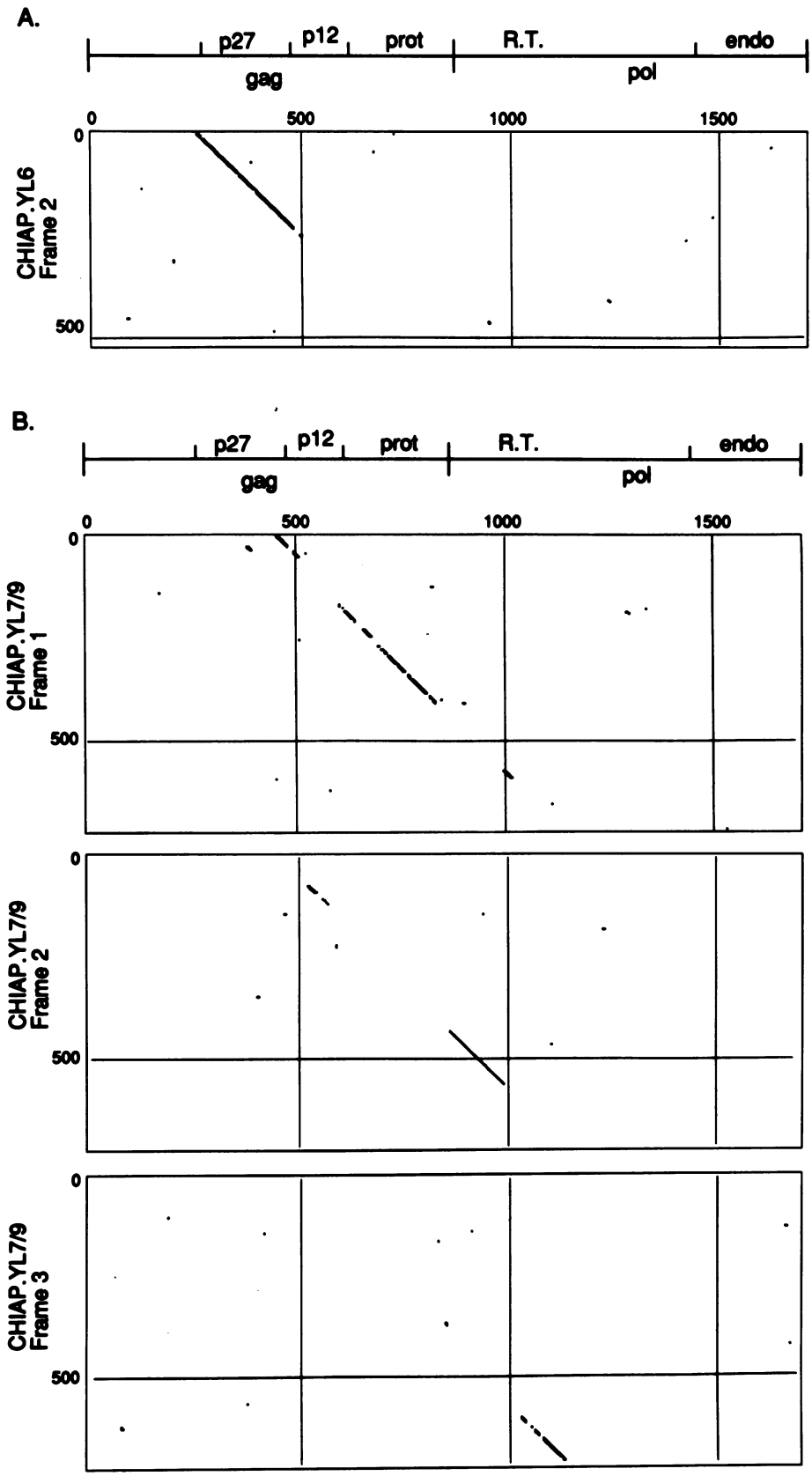

FIG. 8. Amino acid homology of predicted polypeptide sequences of CHIAP cDNA clones CHIAP.YL6 (A) and CHIAP.YL7/9 (B) to the predicted SHIAP H18 *gag-pol* fusion polyprotein. The amino acid sequence of the predicted SHIAP H18 *gag-pol* polyprotein (14, 26) is plotted on the horizontal axis, and the predicted translation products of the indicated frames of the coding strands of each CHIAP clone are plotted on the vertical axes. Each dot of the plot represents 50% amino acid homology over 12 amino acids. Diagonal lines represent regions of significant homology. Multiple frame shifts in the CHIAP.YL7/9 sequence relative to the SHIAP H18 sequence (B) are readily apparent. prot, Protease; R.T., reverse transcriptase; endo, endonuclease.

nome. Nucleotide sequence analysis revealed that these clones are closely related to SHIAP and mouse IAP genes. Significant homologies to MMTV and simian type D viruses were also observed.

The four clones could be segregated into two related but distinct families on the basis of comparisons of nucleic acid sequences. In each family, both members were identical or nearly identical to each other in overlapping regions. However, sequence homology between the two families was less. In a short region in which sequence comparison to the SHIAP H18 genome revealed an overlap between sequences of the two families, only 63% nucleotide identity was observed. In addition, family II sequences showed nearly as much homology to simian type D viruses and MMTV as to SHIAP or mouse IAP genomes, while family I sequences were only distantly related. Servenay et al. (33) recently reported the nucleotide sequence of a 2,016-bp λ clone isolated from a Chinese hamster DNA library which hybridized to a murine IAP probe. This sequence showed homology to the LTR and *gag* regions of the SHIAP H18 genome (72% overall) but was less homologous to family I sequences (64%). Only minimal nucleotide homology to family II sequences (<50%) was present, even though both sequences show homology to an identical region of the SHIAP H18 genome spanning several hundred base pairs. The sequence reported by Servenay et al. may therefore represent a third family of IAP-like sequences in Chinese hamster DNA. The existence of related but distinct families of sequences is consistent with previously published reports suggesting that IAP-related elements in Chinese hamster DNA are relatively heterogeneous compared with IAP genes in mice and Syrian hamsters (18, 19).

Both family I clones contain identical internal deletions when compared with the SHIAP H18 gene. The deletion results in a fusion of putative *gag* and *env* regions of the IAP genome. The same discontinuity was shown to occur in CHO genomic DNA, using PCR amplification. Therefore, the sequence discontinuity undoubtedly reflects a deletion of genomic sequences rather than a splice in the RNA used as the template for the synthesis of cloned cDNAs. Similar internal deletions of genomic DNA have been well documented for murine IAP sequences (reviewed in reference 14).

IAP-related sequences were estimated to occur in the genome of CHO cells at moderately repetitive frequencies (approximately 300 copies per haploid genome). This estimation is in general agreement with frequencies previously reported for the IAP elements of mice, rats, and Syrian hamsters but conflicts with previously published data suggesting that there are relatively few copies of IAP-related sequences in DNA isolated from Chinese hamster liver or from a Chinese hamster lung fibroblast line (18, 19). This discrepancy may reflect differences in techniques, probes, or sources of DNA used.

RNA blot analysis revealed that both family I and family II sequences are transcribed and expressed as abundant RNA in the cytoplasm of CHO cells. The predominant mRNA representing family I sequences was smaller than expected for intact IAP mRNA (2.9 kb compared with 7.2 kb reported for murine IAP RNA or 8.1 kb predicted for SHIAP RNA [13, 26]). The 4.5-kb internal deletion relative to murine or SHIAP genomes observed in our family I cDNA clones yields a predicted size for the resulting transcript of 2.7 to 3.6 kb. The 2.9-kb transcript observed may therefore reflect the internal deletions of encoding genes described above.

One of the two predominant RNA species which hybridized to the family II probe was large enough to represent an intact retroviruslike mRNA (8 to 10 kb for most characterized retroviruses) (40). The presence of this mRNA species is consistent with the lack of sizeable deletions in the family II sequences and suggests that full-length family II genes exist in CHO cell DNA. The origin of the smaller 4.2-kb RNA hybridizing to the family II probe is unknown. The lack of hybridization of the family I probe to the large mRNA detected by the family II probe, even under low-stringency conditions, confirms the separate identities of these two IAP-related families.

An evaluation of the polypeptide encoding potential of all four CHIAP cDNA clones revealed no intact *gag*, *pol*, or *env* ORFs characteristic of retrovirus genomes. IAP particles do not contain IAP-specific envelope proteins (14), and *env* ORFs have not been identified in IAP sequences of other species (22, 26). Therefore, the existence of an intact *env* ORF was not expected. However, the multiple interruptions of *gag* and *pol* ORFs in our cDNA clones is uncharacteristic of IAP sequences isolated from mice and Syrian hamsters in which *gag* and *pol* ORFs with relatively minor disruptions have been identified. Interruptions of the *gag* ORF similar to those observed in family I and family II sequences are also present in the genomic IAP sequence reported by Servenay et al. (33). While none of these CHIAP clones has exhibited the capacity to encode intact IAP *gag* or *gag-pol* polypeptides, it is possible that such transcripts exist within CHO cells. Functional IAP transcripts from only a few intact IAP genes may represent a small proportion of all IAP-related transcripts in CHO cells. The majority of IAP-related RNA in CHO cells, including that represented by the cDNA clones, may be transcribed from nonfunctional pseudogenes. However, efforts to detect polypeptides in CHO cells which specifically react with antisera to structural proteins of B-type or D-type particles, or IAPs, have thus far been unsuccessful (Y. S. Lie and K. P. Anderson, unpublished results).

The lack of IAP polypeptide encoding capacity in CHIAP clones is consistent with the absence of particles in CHO cells with characteristic IAP morphology and localization despite extensive screening by electron microscopy (16, 17). However, the intracytoplasmic A-type particles of CHO cells resemble the immature particles observed in cells infected with closely related B-type and D-type retroviruses (37) as well as the intracellular particles of the IAP-related M432 retrovirus of *Mus cervicolor* (1). In the latter case, the resemblance is striking. Intracellular particles of the M432 virus usually appear as cytoplasmic clusters associated with centrioles in a manner similar to that observed in CHO cells (8, 9, 17). The extensive nucleotide homology (2) and antigenic relatedness (12) of the M432 virus to murine IAPs provides additional evidence that some IAP-related sequences in CHO cells may encode the observed intracellular particles. Further characterization of additional cloned cDNA and genomic IAP-related sequences should help clarify the role of IAP-related genes and transcripts in CHO cells.

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