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Sequences homologous to retrovirus-like genes of the mouse are present in multiple copies in the Syrian hamster genome

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ABSTRACT

The genome of M. musculus contains many copies of DNA sequences homologous to the 35S RNA of intracisternal type-A particles (IAP) (1,2). A major class of IAP genes has been identified and isolated from a mouse library in Charon 4A (3). Cloned mouse IAP genes were used as probes to study homologous sequences in the DNA of other species. Sequences related to mouse IAP genes were detected in the DNAs from a variety of animal cells. DNAs from rat, gerbil, and hamster cells all gave strong reactions which could be localized to discrete restriction fragments on genomic blots. The reaction of Syrian hamster DNA was particularly strong. Fragments derived from different parts of the IAP gene all reacted with Syrian hamster DNA, and the reactive restriction fragments in the Syrian hamster DNA could be ordered with reference to the known restriction map of the IAP genes. The data suggest that sequences related to mouse IAP genes make up a 7 Kb unit in the Syrian hamster genome. Since the majority of the hamster sequences are quite divergent from those in the mouse, the ease with which they are detected suggests that they must be reiterated in the hamster genome.

INTRODUCTION

Intracisternal type-A particles (IAP) are of widespread occurrence in embryonic and transformed cells of Mus musculus (4-6, see refs. in 7) and of other mammals (8-16). Although the IAPs do not have an infectious extracellular phase, individual IAP genes exhibit certain structural features generally associated with integrated retroviruses [i.e., colinearity with the 35S genomic IAP RNA (17) and the presence of terminal repeated sequences (17,18)]. There are no close sequence homologies between the IAPs and representative type-B and type-C viruses of M. musculus (19), but IAPs do share partial sequence homology (2) with a distinctive class of retroviruses endogenous to the Asian murine species Mus cervicolor and Mus caroli (20).

DNA sequences homologous to mouse IAP RNA are reiterated to the extent of 500-1000 copies per haploid genome of both somatic and germ cells of M. musculus (1,2,21). It is not known whether IAP genetic components entered the mouse through infection with a once-competent retrovirus or whether they

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have evolved as cellular genes to their present retrovirus-like form. Only 20-25 copies of the related genes were found in the DNAs of M. cervicolor and M. caroli (2), and these sequences showed a divergence commensurate with the evolutionary separation of these species from M. musculus (2). Thus IAP sequences have apparently existed in the mouse evolutionary line for at least 4-5 million years (22).

As part of an earlier study (1) we tested the DNAs from a limited number of other species for the presence of sequences homologous to mouse IAP genes. These experiments were done using solution hybridization with a cDNA probe which represented only a portion of the IAP genes. We found low levels of homologous sequences in the DNAs of rat and guinea pig, and somewhat higher levels in the DNA of Chinese hamster.

The availability of cloned IAP genes and sensitive blot hybridization procedures has now made it possible to carry out a more thorough study of IAP-homologous sequences in the DNAs of other species. We have identified sequences homologous to mouse IAP genes in DNAs from a variety of animal cells and have localized these to multiple discrete restriction fragments in the DNAs of rat, gerbil, and hamster cells. The reactions were strikingly strong in the genome of the Syrian hamster, and the data suggest that multiple copies of a 7 Kb counterpart of the mouse IAP genetic unit are found there. This study provides the first conclusive evidence that sequences related to a substantial portion or all of mouse IAP genes are widespread in other rodent genera.

MATERIALS AND METHODS

Sources of DNA

High molecular weight DNA was prepared from the indicated cell lines and tissues as previously described (1). Mink lung (CCL64), bat lung (CCL88), raccoon uterus (CCL74), rabbit SIRC (CCL60), and Syrian hamster BHK (CCL10) cells were obtained from the American Type Cell Collection. Syrian hamster DNA was also prepared from the liver and kidney of an animal obtained from the Small Animal Section, NIH. Fisher rat embryo cells were obtained as a primary culture from Microbiological Associates, Bethesda, Maryland. M. Musculus DNAs were prepared from BALB/c myelomas, RPC-20 and MOPC-460D, originally obtained from M. Potter, NCI; from an A/J neuroblastoma tissue culture line N4 (23), and from the liver of a feral mouse (domesticus). Liver DNAs from M. caroli and M. cervicolor were provided by R. Callahan, NCI. DNA was also prepared from the liver of a deer mouse (Peromyscus)

trapped in Maryland. Chinese hamster lung fibroblasts (V-79) and DNA from human placenta were from O. W. McBride, NCI. Rooster liver DNA was from Francine Eden, NCI, and BSC-1 cell DNA from Maxine Singer, NCI. E. coli and λ DNAs were purchased from New England Biolabs. HTC (rat hepatoma) cells were from R. Stellwagen (USC) (12). Guinea pig granulocytic leukemia cells (strain 13) (14) were from Warren Evans, NCI. The gerbil fibroma cell line (10), guinea pig L_2C (strain 2) leukemia cells (9,13), bovine kidney cell line (8) and feline K12C11 cell line were from J. Dahlberg, NCI.

Filter Hybridizations

DNAs were dissolved in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na citrate) at 0.8 μg/μl and 6.25μl of each was spotted onto nitrocellulose filters. After air drying, the DNAs were denatured by wetting the filters in 0.5 N NaOH, 1.5 M NaCl 3 times for 1 minute with drying between. The filters were neutralized in the same way by wetting with 1 M Tris•HCl, pH 7.4, 2 x SSC. The final treatment was with 2 x SSC, followed by air drying and baking for 2 h at 80°C.

Endonuclease digestion of DNAs, electrophoresis on agarose gels, transfer to nitrocellulose membranes, hybridization, and labeling of probes have been described (3). The washing procedure after hybridization of nitrocellulose membranes varied, and is specified in the Figures. In most cases, three washes of 15' each in 0.1 x SSC, 0.25% SDS were done at the temperature indicated. Filters were not allowed to dry between successive washes.

Recombinant DNA clones

Cloned A-particle genes were isolated from a library of mouse DNA in λ phage Charon 4A (generously provided by Jon Seidman, NIH). One recombinant, λMIA14 (3), contains the entire A-particle gene sequence. pMIA1 contains a 5.2 Kb A-particle gene fragment cloned by insertion of a genomic restriction fragment into the plasmid pBR322 (3).

IAP Genomic RNA

Polyadenylated RNA was prepared from neuroblastoma IAPs and fractionated on isokinetic sucrose density gradients (24). The 35S component was subjected to controlled partial hydrolysis with alkali and end labeled with ^{32}P as previously described (3).

RESULTS

A-particle genes occur in the mouse genome as dispersed homologous but non-identical 7 Kb units; a number of these have been isolated from a library of mouse DNA in phage λ and have been characterized in considerable detail

(3,17). A partial restriction map for the 7 Kb IAP genes showing the origin of DNA fragments used as probes in this study is shown in Figure 3.

Hybridization of Mouse IAP Sequences to Heterologous DNAs

Heterologous DNAs were bound to nitrocellulose filters and hybridized with ^{32}P-labeled IAP genomic RNA (6.8 Kb) or with cloned DNA (7.3 Kb). Hybridization was carried out under conditions designed to permit association of divergent sequences. The filters were then washed at increasing temperatures to evaluate the degree of base matching in the hybrids. In this assay the strength of reaction is influenced by sequence content as well as the degree of divergence between the sequences. Figure 1 shows the hybridization of mouse IAP probe to 25 different DNA samples. Results were identical whether the RNA or cloned DNA was used as probe.

After the least stringent wash (Fig. 1A), strong reactions were seen with all *Mus* DNAs. Under these conditions, the strength of reaction with DNAs from the two Asian species, which have diverged from *M. musculus* and contain fewer copies of the IAP-related genes, was not distinguishable from that of *M. musculus* DNA except at very short exposure times (not shown). The only non-*Mus* DNA with a strong reaction was that from Syrian hamster (Fig. 1A, IV-3). Weaker reactions were seen with DNAs from gerbil, rat, guinea pig, monkey, cat, deer mouse, and mink, and still weaker reactions with DNAs from bat, raccoon, and Chinese hamster. The contrasting reactivities of Chinese and Syrian hamster DNAs were noteworthy. No reaction was detected with DNAs from *E. coli*, phage, rooster, or human even after long (68 h) exposure.

After the next wash (Fig. 1B), all DNAs from *Mus* still gave strong reactions as expected, and the reaction with Syrian hamster DNA still persisted although the intensity was reduced. Faint reactions with DNAs from gerbil, rat, guinea pig, cat, deer mouse, and Chinese hamster were still detectable after a short exposure (13h).

After the third wash (Fig. 1C), differences in the degree of reaction of *M. musculus* and *M. caroli* and *cervicolor* DNAs became apparent. We earlier determined that the T_m of the hybrids formed with IAP cDNA and Asian mouse DNAs was 7°C lower than the T_m of the homologous hybrids (2). The reaction with Syrian hamster DNA was still detectable, but quite weak after short exposure (13 h). Other non-*Mus* DNAs which had reacted were barely detectable even after long exposure (48 h., not shown).

The last wash (Fig. 1D) markedly reduced the amount of label hybridized to the Asian mouse DNAs compared with *M. musculus* DNA. At this stringency

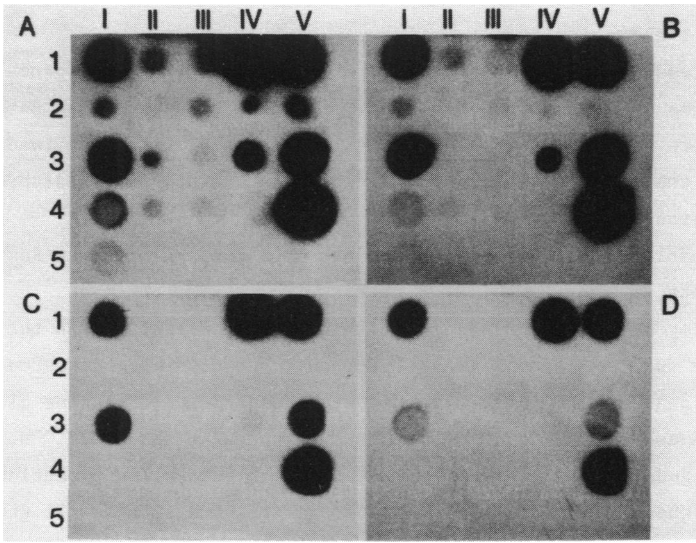

Figure 1. Hybridization of mouse IAP sequences to DNAs from other species. Aliquots (5μg) of DNA were spotted on nitrocellulose filters and the filters hybridized with 4 x 10^5 cpm/ml of ^{32}P-labeled kinased IAP 35S RNA for 46 h as previously described (3). Panels A-D show autoradiograms after washing under conditions of increasing stringency. A. The filter was washed in 50% formamide, 4 x SSC at 37°C. B. The filter was washed in 0.1 x SSC, 0.25% SDS at 55°C. C. The filter shown in A was rewashed in 0.1 x SSC, 0.25% SDS, at 60°C. D. The filter shown in B was rewashed in 0.1 x SSC, 0.25% SDS, at 65°C. The filters were exposed to film for 13 h. Sources of DNA are indicated below; + or - in parentheses indicates whether or not cells contain intracisternal virus-like particles.

I-1	*M. musculus* neuroblastoma N4	(+)	III-1	Rat embryo	(-)
I-2	Guinea pig L_2C leukemia	(+)	III-2	Monkey BSC-1 cells	(-)
I-3	*M. caroli* liver	(-)	III-3	Bat lung	(-)
I-4	Rat hepatoma	(+)	III-4	Raccoon uterus	(-)
I-5	Chinese hamster with three copies of mouse IAP genes per cell	(-)	III-5	*E. coli*	(-)
II-1	Gerbil fibroma	(+)	IV-1	*M. musculus* myeloma 460D	(+)
II-2	Bovine embryo kidney	(+)	IV-2	Feline K12C11	(+)
II-3	Mink lung	(-)	IV-3	Syrian hamster-BHK cells	(+)
II-4	Guinea pig granulocytic leukemia	(+)	IV-4	Chinese hamster	(-)
II-5	Human placenta	(-)	IV-5	λ phage	(-)

V-1	*M. musculus* myeloma RPC20	(+)
V-2	Deer mouse liver	(-)
V-3	*M. cervicolor* liver	(-)
V-4	*M. domesticus* liver	(-)
V-5	Rooster liver	(-)

the reaction with Syrian hamster DNA was detectable only after long (68 h) exposure (not shown).

These results indicate that sequences related to mouse IAP genes are present in the DNAs from a variety of animal cells. For the most part the reactions were weak as expected from the evolutionary distance between these species and the mouse. The Syrian hamster DNA, however, gave a disproportionately strong reaction.

Sequences Homologous to Mouse IAP Genes Are Localized in Discrete Restriction Fragments in the DNAs of Other Species

Heterologous DNAs which showed detectable hybridization with the IAP probe on the spot test were further examined. The DNAs were digested with *Hind*III and analyzed by the Southern blotting technique (25) using cloned A-particle DNA as probe (Fig. 2). The least stringent wash was 26°C below the T_m of homologous mouse hybrids (designated T_m -26°) to allow detection of divergent sequences. Discrete reactive fragments were seen in all the DNA samples although their number and intensity varied a great deal (Fig. 2A). A few weakly reactive fragments were seen in the DNAs of monkey, cat, mink, and raccoon. A small number of slightly stronger fragments were present in those of guinea pig and deer mouse. Gerbil DNA contained a large number of discrete fragments. Syrian hamster, Chinese hamster, and rat DNAs all contained multiple fragments which hybridized strongly under these conditions. None of the discrete reactive fragments in these DNAs corresponded in size to the prominent *Hind*III fragments in mouse DNA (see Fig. 1 ref. 3 and Figs. 6 and 7 ref. 13). Because the reaction of the Syrian hamster DNA on the spot test in Figure 1, isolated from cultured cells, was so much more intense than that for Chinese hamster, we considered the possibility that DNA from BHK cells was not representative of Syrian hamster germ line because of changes which might have occurred during prolonged propagation in culture. In the experiment shown in Figure 2, we used DNA prepared from the liver of a Syrian hamster.

After a more stringent wash at T_m-14° (Fig. 2B), the only discrete fragments which reacted strongly were those in the DNAs of Syrian hamster, Chinese hamster, and rat, and after an even more stringent wash at T_m-9° (Fig. 2C), the only strongly reactive fragment which remained was a 2.3 Kb fragment in Syrian hamster DNA.

Sequences from All Parts of the IAP Genome Are Represented in Syrian Hamster DNA

The reactions of Syrian hamster, Chinese hamster and rat DNAs in Figure 2

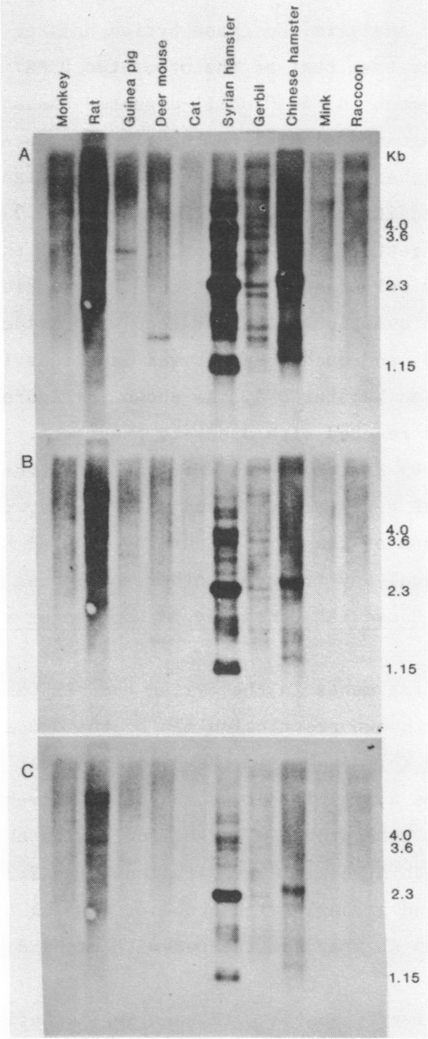

Figure 2. Identification of sequences homologous to mouse IAP genes in restriction fragments in the DNAs of other species. Aliquots of the indicated DNAs (20 μg) were digested with HindIII, separated by electrophoresis in a 1.2% agarose gel, transferred to nitrocellulose filters and hybridized at 50°C for 23 h with a ^{32}P-labeled DNA fragment (probe e, see Fig. 3) which represents most of the IAP gene. The filter was washed in 0.1 x SSC, 0.25% SDS, at increasing temperatures and exposed to film for 18 h after each wash. A. Washed at 43°C (calculated T_m-26°); B. Washed at 55°C (T_m-14°); C. Washed at 60°C (T_m -9°). The theoretical T_m was calculated using the formula (26) : $T_m = 16.6 \log (Na^+) + 0.41 (\% G+C) + 81.5$. Rat DNA was from hepatoma HTC cells (12) and guinea pig DNA was from the granulocytic leukemia cells (14).

indicated that there were enough IAP-related sequences present in discrete fragments for further analysis. We chose Syrian hamster DNA because the reaction was much stronger than that of the other two DNAs. The presence of a prominent 2.3 Kb fragment in the HindIII-digested DNA as well as many less intensely labeled fragments also suggested that sequences related to some portions of the IAP genes might be present in the Syrian hamster genome in larger amounts. In addition, the strongly reacting 2.3 Kb fragment survived even relatively stringent (T_m-9°) washing, suggesting that some sequences might be particularly well conserved between this species and the mouse.

To examine these questions, the IAP gene was divided into regions as indicated in Figure 3, and each fragment was used individually as probe with HindIII-digested Syrian hamster DNA. As shown in Figure 3, probes from all parts of the IAP gene reacted with discrete fragments. The strongest reactions (the 2.3 and 1.65 Kb fragments) were seen with the probes derived from the 3' half of the IAP gene (Probes c and d). Probe e, containing the sequences in a-c plus regions of the 5' terminus and about 1 Kb of the 5' flanking sequence, did not reveal any fragments other than those seen with a-c.

Sequences Related to Mouse IAP Genes May Be Located on a 7 Kb Unit in the Syrian Hamster Genome

The restriction fragments in the Syrian hamster DNA could be ordered with reference to the known restriction map of the mouse IAP genes as shown in Figure 3. The 1.8 Kb fragment in the HindIII digest of Syrian hamster DNA reacted with probe a, placing it as the most 5' fragment. The 1.15 Kb fragment reacted with both probes a and b, indicating that sequences in this fragment span both A-particle gene regions. The 2.3 Kb fragment reacted weakly with probe b and strongly with probe c, placing it mostly in the region of c. The 1.65 Kb fragment reacted with probe d, making this the 3' most fragment.

Additional reactive fragments were seen in the Syrian hamster genomic blots (Fig. 3). A fragment of 3.6 Kb reacted with probes b and c, while a 4 Kb fragment reacted with probes c and d (and weakly with b). These and other larger fragments are most easily accounted for by assuming that restriction site variants missing one or more of the internal sites exist (Fig. 3); similar types of variants in internal restriction sites have been found among the multiple IAP genes in the mouse genome (3,17). Other less prominent bands may reflect other types of polymorphism in the homologous sequences; a similarly complex array of fragments is also seen in certain mouse genomic digests (3,17). If we assume that the reactive 1.8, 1.15,

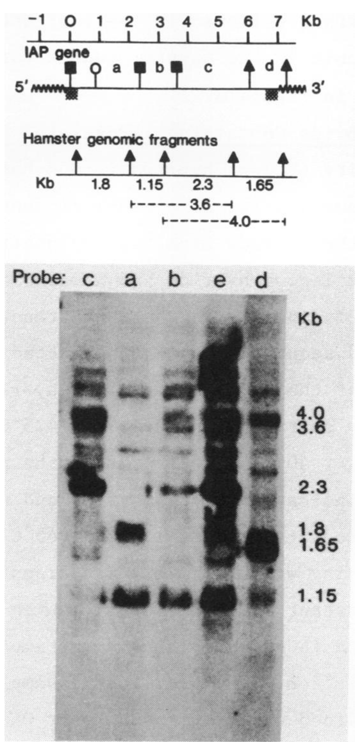

Figure 3. Sequences from all parts of the IAP gene react with Syrian hamster DNA. A partial restriction map of the 7.3 Kb IAP gene is shown at the top; the ends of the gene are delineated by the long terminal repeats (LTRs), ▩, and mouse flanking sequences are shown as ⩘⩘⩘. Fragments used individually as probes a-d are shown on the map. Probes a-c were prepared from pMIA1 (3) which contains IAP sequences from 0.8-6.0 map units and was derived by insertion of an EcoRI/HindIII genomic DNA fragment into pBR322. The plasmid was cut with PstI and PBR322 sequences were left attached to the end fragments (probes a and c) to facilitate separation. Probes d and e were prepared from λMIA14 (3), containing the entire A-particle gene in Charon 4A phage, by digestion with HindIII. Fragment d has been subcloned in pBR322 (designated pMIA6), and shown to contain the 3' LTR (17). Probe e extends from the HindIII site at 6.0 map units to a HindIII site at -1.1 in the 5' flanking region. Although probe e lacks the 3' LTR and a small amount of sequence immediately inside the 3' LTR, it is representative of essentially the entire gene since the 5' LTR is present. All fragments were recovered individually from an agarose gel by binding on glass microbeads and nick-translated for use as probes as previously described (3). Each probe was hybridized separately with a blot of 20 μg of HindIII digested Syrian hamster liver DNA. The blots were washed in 0.1 x SSC, 0.25% SDS, at 55°C. The restriction fragments detected with each probe were ordered with reference to the known restriction map of the IAP genes, and the structure of the postulated 7 Kb genetic unit in the Syrian hamster genome is indicated above the blot. The possible position of larger fragments resulting from putative restriction site variants is shown by broken lines. Restriction sites: ▼, PstI; ♀, EcoRI; ↑, HindIII.

2.3, and 1.65 Kb fragments are linked in the indicated order, they could make up a 7 Kb genetic unit in the hamster genome for which a postulated structure is diagrammed in Figure 3.

Thermal Stability of Hybrids Containing IAP-Related Sequences

The thermal stability (T_m) of hybrids formed between a mouse IAP probe and DNAs from heterologous species at a non-stringent temperature was measured by elution of the hybrids from hydroxylapatite columns. Mouse DNA was used to form the homologous hybrids and human DNA was used to assess the extent of non-specific binding. With the short reaction used to form the hybrids, only reiterated sequences would be expected to react.

Figure 4A shows that the melting curve for hybrids formed between probe c and mouse DNA was very sharp and had a T_m of 84.5°C, very close to the expected T_m of 85.1°C. At 40°C at least 70% of the probe was bound with all the heterologous DNAs, indicating that specific and non-specific binding could not be distinguished at this temperature (45°C below the T_m of homologous hybrids). The binding with human DNA was dramatically reduced with the next two washes to a constant fraction (~15%), which probably represented self-annealed probe since it melted at the same temperature as authentic mouse hybrids. This fraction was higher in the sample hybridized with human DNA than in those hybridized with rat and hamster DNAs where part of this material was incorporated into hybrids, and therefore was not available for self-annealing.

The percent of probe bound by hamster and rat DNAs over this background binding with human DNA is shown in Figure 4B. Syrian hamster DNA bound more probe than the Chinese hamster and rat DNAs. At 58.5°C, corresponding to T_m-26°, Syrian hamster DNA bound twice as much label as Chinese hamster and rat DNAs. However, at 75.5°C (T_m-9°), there was no detectable increment in label bound by Syrian hamster DNA over the other DNAs even though in the blot in Figure 3C the Syrian hamster DNA gave a much stronger reaction. The T_ms of the hybrids formed with the two hamster DNAs and the rat DNA were virtually identical. In addition, the hybrids all melted at a temperature (T_m 61-62°C) which indicated that most of the IAP-related sequences are quite divergent from those of the mouse. Thus, since there was no difference in T_ms of the hybrids, and no increment in amount of probe bound to the column, the much stronger hybridization of Syrian hamster DNA with IAP probe under stringent conditions of washing (T_m-9°C, Fig. 2C) is probably due to a higher reiteration frequency of the IAP-related sequences in that species.

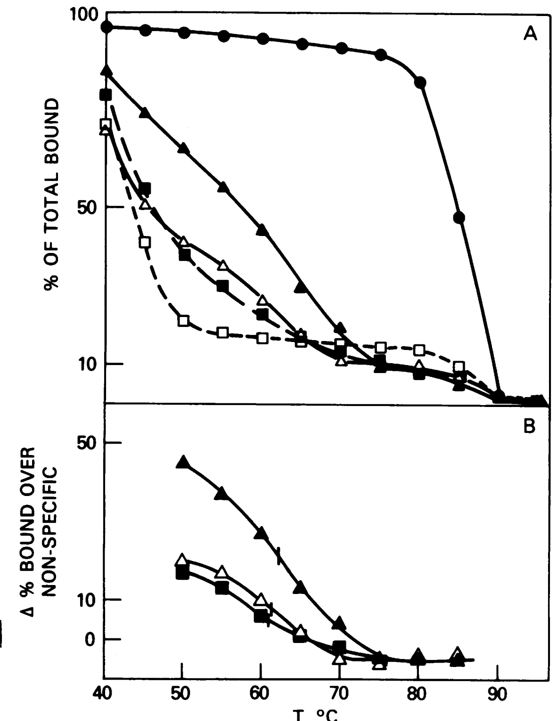

Figure 4. Thermal stability of hybrids containing IAP-related sequences. A. Probe c (with pBR322 sequences removed) labeled with ^{32}P was hybridized with 80 μg of sheared cell DNA at 50°C to a C_0t of 54. Time of hybridization was 1 h to minimize self-annealing of the double stranded probe. For chromatography on hydroxylapatite, the samples were diluted and loaded on the column in 0.12 M sodium phosphate (1:1) at 40°C. Three 1 ml fractions were eluted at each temperature at 5° increments. The cellular DNAs were as follows: ●, M. musculus (Swiss liver); ▲, Syrian hamster (kidney); Δ, Chinese hamster (V-79 cells); ■, Rat (HTC cells); □, human (placenta). B. The percentage of probe bound by rodent DNAs over background binding with human DNA. Vertical lines indicate the T_m of each hybrid.

DISCUSSION

In this study we have shown that sequences related to mouse IAP genes are present in the DNAs of rat, gerbil, and hamster cells. We have characterized the sequences in the Syrian hamster in some detail. The Syrian hamster genome contains sequences homologous to all parts of the mouse IAP genes (Fig. 3), but the strongest reaction in genomic digests was given by a 2.3 Kb HindIII fragment. This fragment hybridized with IAP probes b and c, particularly the latter. A strong reaction might be expected either if the

hamster sequences were highly conserved with respect to the mouse or if these sequences (even though diverged) were highly reiterated. The hybrids formed between Syrian hamster DNA and IAP probe c had a ΔT_m of -20°C to -25°C with respect to homologous hybrids between the probe and mouse DNA (Fig. 4). This ΔT_m indicates a considerable divergence and suggests that the 2.3 Kb HindIII segment must be extensively reiterated in the Syrian hamster genome in order to give such strong hybridization in Southern blot experiments.

Our results eliminate the possibility that A-particle sequences entered the Syrian hamster genome as a result of relatively recent infection of animals with a mouse virus. The divergence of the IAP-related sequences in the Syrian hamster genome from those of the mouse indicates that these sequences have existed in the Syrian hamster for millions of years. This observation is important because all laboratory stocks of the Syrian hamster originated from breeding 3 litter mates (27), and we have no experience with feral animals.

The reactive restriction fragments in the Syrian hamster genome could be ordered into a 7 Kb counterpart of the mouse IAP genetic unit (Fig. 3). We speculate that such reiterated genes code for the Syrian hamster intracisternal virus-like particles which are expressed in embryonic and tumor cells (11, 28) in a manner analagous to the expression of mouse IAPs. Restriction patterns for DNAs from Syrian hamster cells which express virus-like particles (BHK) and from cells which do not express particles (liver) were identical; we have also found this to be the case for DNAs from IAP-positive and negative mouse cells (17).

The strength of reaction of the genomic DNAs with IAP probe could not be directly correlated with whether or not the cells express virus. A number of other non-mouse cell lines tested also express intracisternal virus-like particles [rat hepatoma (12), guinea pig leukemias (9,13,14), gerbil fibroma (10), bovine kidney (8), and the feline K12C11 cells (J. Dahlberg, personal communication)] but did not hybridize strongly to the mouse IAP probe. Typical intracisternal virus-like particles have not been observed in Chinese hamster cells, but some cells from this species do contain intracytoplasmic particles which morphologically resemble particles in mouse cells infected with the IAP-related M432 virus of M. cervicolor (29).

IAP-related sequences are found in the cellular DNA of all species of the genus Mus (30) and in the case of M. cervicolor and M. caroli, sequences homologous to the entire IAP genome are present (2). A similar situation

seems to be the case for a more distantly related rodent, the Syrian hamster. Thus sequences homologous to IAP genes are present in two families of rodents, Muridae and Cricotidae, which diverged 20 million years ago (31). There is some suggestion that homologous sequences may also be present in a more distantly related rodent, the guinea pig, as well as members of other mammalian orders.

We have shown that the IAP probe could be used to identify related genetic elements in gene libraries prepared from other species. This would permit us to determine if the IAP-related sequences in those DNAs also represent retroviral genes.

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