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The expression of intracisternal A particle genes in the mouse preimplantation embryo

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The Expression of Intracisternal A Particle Genes

in the Mouse Preimplantation Embryo

by

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DEDICATION

To my parents, Jean Frances Chambers Poznanski and Walter Antonovitch Poznanski, for all their love and support.

The Expression of Intracisternal A Particle Genes in the Mouse Preimplantation Embryo

Ann Alexandra Poznanski

Abstract

The work presented in this thesis describes the normal expression of a family of endogenous retroviral genes, the intracisternal A particle (IAP) genes, during preimplantation development of the mouse, *Mus musculus*. There are approximately 1000 copies of intracisternal A particle genes in the genome of *Mus musculus* , comprising about 0.3% of the murine genome. Two subfamilies have been distinguished and termed as type I and type II. I have examined the expression of IAP sequences in embryos in three ways. First, I have examined IAP-related proteins by protein blot analysis and by immunoprecipitation using antibodies to components of the intracisternal A particle, primarily the *gag* or core protein. Secondly, I have examined IAP-related transcripts by RNA slot blot and Northern analysis using type I and type II specific IAP DNA probes. Finally, I have examined type I and type II IAP transcripts by amplification of embryo cDNAs using specific oligonucleotide primers in the polymerase chain reaction (Saiki et al., 1985, 1988). The results of these experiments demonstrate that the IAP core protein, p73, is present throughout preimplantation development from the unfertilized egg through the blastocyst stage and the IAP-related p120 is present at the 8-cell and blastocyst

stages. Both type I and type II IAP genes are transcribed at all stages examined and the rate of transcription is similar on a per cell basis for the 8-cell and blastocyst stage embryos. The PCR experiments reveal that the type I PCR product of 417 base pairs (bp) is present at all stages, from the unfertilized egg through blastocyst stage, while the presence of the two type II PCR products varies according to stage. Both type II PCR products, the 242 bp product and the 292 bp product, are present in the unfertilized egg, the 8-cell embryo and the blastocyst. At the 2 cell stage, when formed particles are abundant, only the smaller product of 242 bp is present. The research which comprises this thesis was undertaken in an effort to examine whether any of the elements of heterogeneity in the expression of IAP sequences correlates with the burst of particle production seen at the 2-cell stage. What I have found is almost the reverse. The 2-cell embryo lacks both an IAP-related p120 and one of the type II transcripts which can be distinguished by my PCR strategy.

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INTRODUCTION

The work presented in this thesis describes the normal expression of a family of endogenous retroviral genes, the intracisternal A particle (IAP) genes, during preimplantation development of the mouse, *Mus musculus*. This research has as its origin an observation made during the course of an EM survey of preimplantation embryos. In this ultrastructural study Calarco and Brown (1969) noted that at the two cell stage the endoplasmic reticulum (ER) surrounding the nuclei is filled with particles which have a virus-like appearance. At stages prior to or after the 2-cell stage these particles are scarce or absent. This observation was expanded through further studies to include many different strains of mice, both inbred and feral (Calarco, 1975, 1979; Yotsuyanagi and Szollosi, 1981, 1985; Chase and Piko, 1973). The appearance of these particles during early development has also been noted in two Asian mouse species, *Mus pahari* and *Mus cervicolor* (Calarco et al., 1980; Yotsuyanagi and Szollosi, 1984). In all strains of mice which have been examined the appearance of these virus-like particles has been shown to be part of the normal program of preimplantation development.

The virus-like particles are between 70 and 100 nanometers in diameter and consist of two concentric shells surrounding a relatively electron lucent core and are similar in morphology to particles found abundantly in numerous mouse tumors and neoplastic cell lines. Formation of the particles occurs first by

accumulation of protein in electron dense patches on the cytoplasmic surface of the rough ER in regions without ribosomes and then spherical buds intrude into the cisternae of the ER or into the nuclear envelope. The particles are never found extracellularly. IAPs isolated from tumor cells are not infectious (Hall et al., 1968; Kuff et al., 1968). Because these particles are characterized by their exclusive localization within the cisternae of the endoplasmic reticulum, they have been named intracisternal A particles (IAPs) (PCNV, 1966). Intracisternal particles have also been observed in the germ cells and embryos of several other species including guinea pig (Enders and Schlafke, 1965; Andersen and Jeppesen, 1972: Black, 1974; Fong and Hsiung, 1976) hamster (Bernhard and Tournier, 1964; Sobis and Vandeputte, 1978) and cat (Bowen, 1980).

Intracisternal A particles were first isolated from mouse plasma cell tumors by Kuff, Wivel and Lueders (1968). Biochemical and molecular analysis of the isolated particles revealed that they possess a high molecular weight polyadenylated RNA genome (Yang and Wivel, 1973, 1974; Wong-Staal et al., 1975; Lueders and Kuff, 1977), a core protein of 73 kD (Wivel et al., 1973) and a Mg++ dependent reverse transcriptase (Wilson and Kuff, 1972) distinct from the C-type retrovirus enzyme. The outer shell of the particle was found to be composed entirely of ER membrane. The cloning of the RNA genome of the particles, and the use of this cloned genome as a probe (Lueders and Kuff, 1977), revealed that these particles are encoded by members of a large family of endogenous proviral genes

which are present in the genome of all mice and are distributed among most, if not all, chromosomes.

The origin of endogenous retroviruses in vertebrate genomes is not known. It has been suggested that these sequences arose either from an alteration and evolution of cellular genes (Temin, 1974) or by infection of the ancestral germline (Todaro et al., 1975). The IAP sequences in mice show a divergence commensurate with the evolutionary separation between species of mice (Kuff et al., 1978). These sequences are estimated to have been present in mice for at least 4-5 million years (Lueders and Kuff, 1981).

IAP Element DNA Structure

There are approximately 1000 copies of intracisternal A particle genes per haploid genome of Mus musculus. These copies are non-identical but homologous. This comprises about 0.3% of the murine genome. All retroviruses share a set of common features. Based upon ultrastructural criteria murine retroviruses have been classified into three types: A, B, and C (Bernhard, 1958). The retroviral particles are similar in overall morphology. They are spherical particles of 70-100 nm in diameter and contain a nucleoprotein core of 30-70 nm. Their outer layer consists of unit membrane which is acquired in the process of particle formation during budding, either through the plasma membrane, into the cisterna of the endoplasmic reticulum or into the space between the inner and outer nuclear membranes. Their genomes of

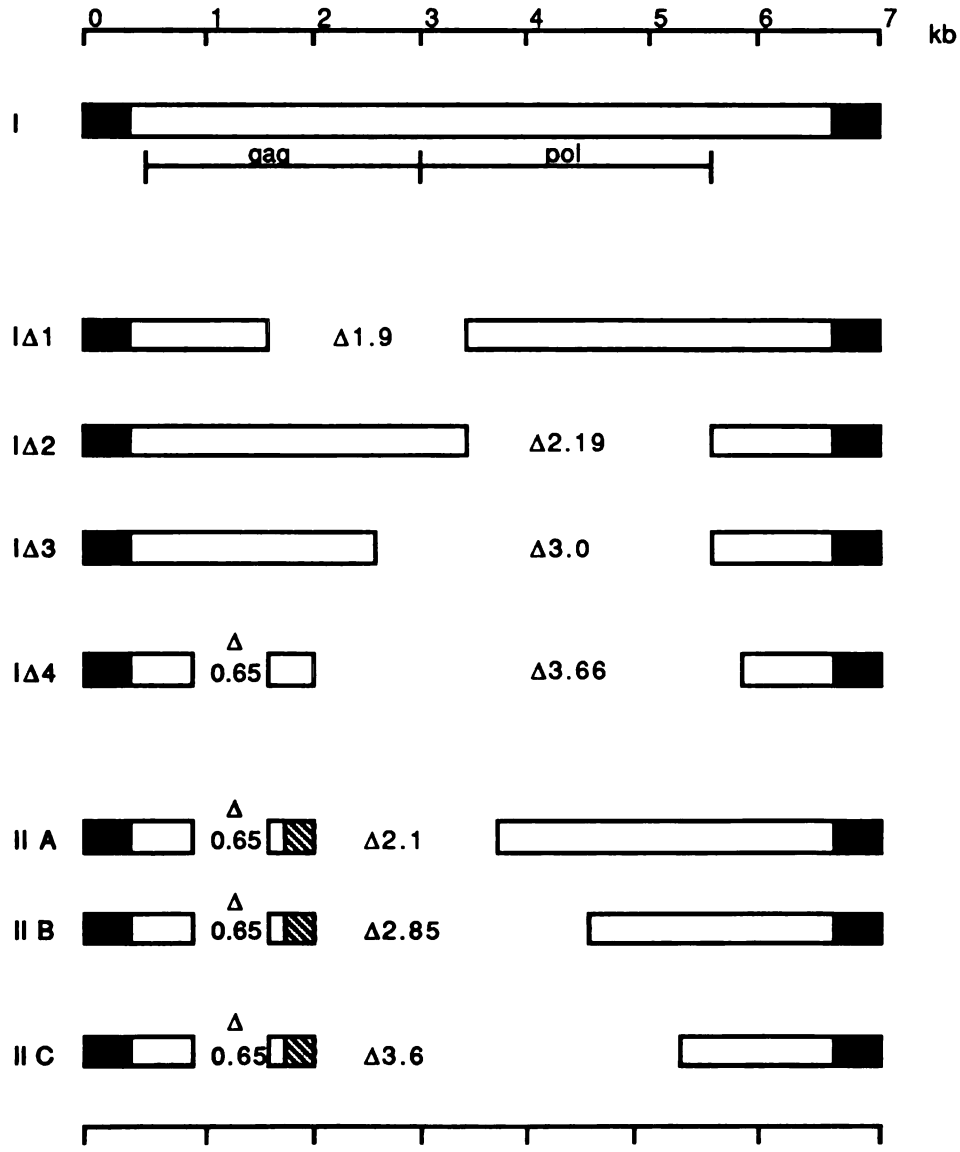
approximately 8-10 kilobases (kb) are diploid, consisting of two subunits of single stranded RNA. Sequence analysis of the integrated proviral DNA has allowed fine structural mapping of the retroviral genome and identification of functionally important domains. The prototypical retrovirus genome codes for three major viral proteins. The genes for these proteins are abbreviated as *gag*, *pol* and *env*. The *gag* (group specific antigen) genes codes for the major core protein. It is translated as a polyprotein precursor which is processed by cleavage into several species. The *pol* gene codes for the reverse transcriptase enzyme, an RNA-dependent DNA polymerase. The *env* gene codes for the envelope glycoprotein which is incorporated into the outer unit membrane of the retrovirus particle. In common with both proviral DNA of typical retroviruses and transposable elements of bacteria, yeast and Drosophila, the integrated DNA form of IAP sequence contains terminally repeated sequences (Cole et al., 1981; Kuff et al., 1981). The IAP sequences do not share any significant homology with either type B or type C viruses (Lueders and Kuff, 1979) but show some sequence homology with a unique endogenous virus of Asian murine species, *Mus cervicolor* and *Mus caroli* (Kuff et al., 1978; Callahan et al., 1981).

Two subfamilies of IAP genes have been distinguished and termed as type I and type II (Fig. 1). Type I genes include the full length gene of 7 kb and several deleted versions. These comprise about 60% of all IAP genes (Lueders and Kuff, 1980; Lueders and Meitz, 1986). Type II genes are distinguished in two ways from type I genes. They contain major deletions involving the *gag* and *pol*

genes and they contain a unique inserted sequence of approximately 270 base pairs (Sheng-Ong and Cole, 1982; Lueders and Meitz, 1986) (indicated by the hatched box in Fig. 1). Type II genes comprise about 40% of all IAP genes (Lueders and Meitz, 1986).

Fig. 1. Structure of mouse IAP elements (adapted from Kuff and Lueders, 1988). The major genetic regions of a full size type I IAP element is shown at the top. IAP internal sequences are represented by open boxes, LTRs by solid boxes. Four classes of type I deleted elements are shown beneath with Δ indicating the size in kilobases of the missing sequence. Three classes of type II IAP elements are shown; the typeII-specific insertion, AIIins, is indicated by a hatched box.

IAP Genes


0
1
2
3
4
5
6
7
kb
I
gag
pol
IΔ1
Δ1.9
IΔ2
Δ2.19
IΔ3
Δ3.0
IΔ4
Δ
0.65
Δ3.66
II A
Δ
0.65
Δ2.1
II B
Δ
0.65
Δ2.85
II C
Δ
0.65
Δ3.6

IAP-related Proteins

IAP-related proteins have been identified as components of isolated particles and as proteins immunoprecipitated by anti-IAP antisera from IAP-producing cells. Particles are isolated by mechanical shearing in the presence of Triton X-100 and isopycnic banding through sucrose gradients (Kuff et al., 1968). A 73 kD protein (p73) has been identified as the main structural protein of IAPs isolated from cells of three myelomas and one neuroblastoma. This *gag*-related protein is equivalent to the uncleaved *gag*-precursor proteins of conventional retroviruses. In addition proteins of three other molecular weights (100 kD, 45 kD, 35 kD) have been isolated as minor components (Marciana and Kuff, 1973, 1974). These proteins share tryptic peptides in common with p73, but their metabolic relationship to p73 is not clear.

The *gag*-related core protein, p73, is difficult to solubilize. It is characteristically insolubile in the presence of excess Triton X-100 and EDTA or other detergents such as NP-40 or deoxycholate and solubilization requires the addition of SDS or guanidine thiocyanate (Lueders and Kuff, 1975). In a kinetic labeling study (Lueders and Kuff, 1975) it was shown that p73 and the three minor proteins are rapidly labeled (within 2 minutes) and incorporated into particles, as judged by their shift into a Triton X-100/EDTA insoluble fraction. No cytoplasmic pool of p73 was detected. IAP proteins are synthesized on membrane bound polyribosomes, remain entirely intracellular

and represent 1.2% of the protein synthesis in neuroblastoma cells (Lueders, 1976).

A larger protein of 114 kD to 120 kD (p120) has been identified as an IAP-related protein by its immunoprecipitation by an anti-IAP antisera (A1.3) from IAP-producing cells (Kuff and Fewell, 1985). Because a significant portion of this protein can be solubilized with Triton X-100 or NP-40 it has been suggested that the p120 may not be associated with particles but rather exists in a cytoplasmic pool (Kuff and Lueders, unpublished observations).

The p73 and p120 have also been shown to be the in vitro translation products of IAP RNAs. The p73 can be translated from an IAP RNA of 7.2 kb and the p120 can be translated from an IAP RNA of 5.4 kb (Patterson et al., 1978; Kuff and Fewell, 1985).

IAP-related RNAs

Polyadenylated RNAs which range in size from 28S to 35S have been found to be associated with IAPs. The RNAs which are present vary according to the cells from which they are isolated. From the N4 neuroblastoma cell two major IAP-related RNAs can be isolated (Patterson et al., 1978), a 35S RNA which corresponds to a 7.2 kb type I transcript and a 32S RNA which corresponds to a 5.4 kb type IΔ1 transcript. In MOPC-104E and MOPC-315 plasmacytoma cells the main IAP-related RNA is a 28S species which corresponds to a 4.0 kb transcript of a type IIB gene (Patterson et al., 1978; Lueders

et al., 1977; Sheng-Ong and Cole, 1982). Minor amounts of IAP-related RNAs of 4.7 kb (type IIA) and 3.5 kb are also found in these cells. By contrast, another plasmacytoma cell line, MOPC-21, has predominately the 7.2 kb and 5.4 kb transcripts with very little type II RNA (Kuff and Fewell, 1985). The ability of the 7.2 kb and 5.4 kb transcripts to code for the IAP-related proteins p73 and p120 was suggested by the correlation between the abundance of these species in the thymus of various mouse strains (Kuff and Fewell, 1985). In addition, it has been shown that these transcripts can encode the corresponding proteins in an in vitro translation assay (Patterson et al., 1978). The open reading frames of sequenced IAP genes predicts proteins of this size as well (Kuff et al., 1986b).

In addition to the single stranded IAP-related RNAs, one group has reported that double stranded IAP-related RNA has been found to comprise 0.004% of the heterogeneous nuclear RNA of Ehrlich ascites carcinoma cells (Kramerov et al., 1985). Free supercoiled circular DNA molecules homologous to IAP genes have also been found in the same cells (Grigoryan et al., 1985). The authors suggest that these molecules are intermediates in the process of integration of new provirus into the genome.

Immunoglobulin Regulatory Factors Encoded by Members of the IAP Family

In the course of experiments undertaken to isolate cDNA clones that encode rodent IgE binding factors (IgE-BF), Martens et al. (1985)

succeeded in isolating and expressing clones from a rat-mouse T cell hybridoma that turned out to be members of the IAP family (Moore et al., 1986). The factors expressed by these clones are encoded entirely within the mouse IAP sequences. Only a small subset of IAP transcripts actually encodes these proteins which act as IgE-BFs in the biological assay which they used. Four of seventy cross hybridizing cDNA clones were eventually identified as able to encode these factors (Martens et al., 1985). The four clones which have been analyzed by heteroduplex and restriction enzyme mapping were found to represent different structural variants of the full-size IAP genomic elements (Kuff et al., 1986b), homologous throughout the *gag*-related regions. Two fully sequenced clones (8.3 and 10.2) each specify different *gag-pol* fusion peptides, with the open reading frames spanning deleted sequences. The 8.3 clone has an open reading frame of 556 amino acids encoding a 60 kD secreted IgE BF. Another clone (10.2) is a type IΔ1 IAP element encoding a 116 kD peptide, which can presumably be proteolytically cleaved to yield the 60 kD IgE binding factor which is produced by COS7 monkey kidney cells transfected with this sequence.

Antisera made to ovalbumin conjugates of peptides whose sequence was predicted from the 8.3 clone were able to immunoprecipitate IAP-related proteins p73 and p120 from IAP-producing neoplastic cell lines (Kuff et al., 1986b), and an anti-p73 antibody (A1.3) is able to react with IgE-BF in a biological assay (Moore et al., 1986), confirming a relationship between these proteins and characterized IAP proteins. One possibility is that, in

the mouse, during the course of evolution IAP genes have incorporated cellular information that when combined with sequences from the *gag*-coding region can produce proteins that are able to bind to IgE (Kuff et al., 1986b).

IAP Expression in Somatic Cells

Among normal somatic cells, IAP expression is highest in the thymus of young mice (Kuff and Fewell, 1985). Kuff and Fewell have examined the patterns of expressed IAP transcripts in the thymus and found that the number of different transcripts which are expressed and their proportions relative to one another vary according to the strain of the mouse. For instance, in BALB/c thymus approximately equal amounts of the 7.2 kb and 5.4 kb IAP transcripts are expressed at relatively high levels while in C57BL/6 thymus the 5.4 kb transcript is expressed at a lower level and the 7.2 kb transcript is undetectable. Genetic background, therefore, seems to play a role in determing the expression of IAP sequences. There is also, however, evidence for the selective activation of IAP sequences in this tissue (Grossman et al., 1987). Active IAP transcripts were cDNA cloned from the thymuses of two different, distantly related mouse strains (C57BL/6 and DBA/2J). When the cDNAs were sequenced they were found to exhibit a high degree of similarity over a 2.2 kb range. The average diversity among 5 IAP sequenced clones within the same region was calculated as 3% while the diversity between the thymus-derived clones was only 0.3%. This close resemblance was seen as evidence that the same or allelic

IAP genes may be activated in the thymuses of these two mouse strains. The authors suggest that this selective activation could be due to either 1) the action of trans-acting regulatory factors on specific IAP sequences or 2) the specific location of these IAP genes with respect to a regulatory locus and/or segment of "active chromatin" in thymus cells (Grossman et al., 1987).

IAP Expression in Teratocarcinoma Cells

Teratocarcinoma cell lines have been used by many investigators as model systems for the study of aspects of differentiation in early mouse embryos (Martin, 1975). However, there seems to be no consistent pattern for the appearance or absence of IAPs in undifferentiated teratocarcinoma stem cells (Pierce et al., 1967; Lehman et al., 1974; Spense et al., 1975; Lasneret et al., 1978; Canivet et al., 1980) or in their differentiated derivative cells (Teresky et al., 1974; Nicholas et al., 1976; Morgan and Huang, 1987). In some cases IAPs are numerous only in the stem cells (Spense et al., 1975) while in other cases they appear only after differentiation has been induced (Morgan and Huang, 1987) or after several generations of transplantation (Damjanov and Solter, 1973). In fact, different groups of investigators have found contradictory results when examining the same teratocarcinoma cell lines for the presence of IAPs (e.g. Hojman-Montes de Oca et al., 1983 and Moshier et al., 1985). A similar confusion exists when attempts have been made to correlate the presence or absence of IAPs in stem cells or their derivatives with the state of methylation of the IAP genome

as a whole (Hojman-Montes de Oca et al., 1983) or of particular sequences (Morgan and Huang, 1984, 1987). Perhaps it is not surprising that no generalizations are possible in comparing what, after all, are quite heterogeneous cells. The variables which contribute to this heterogeneity include the genetic background of the teratocarcinoma cell lines, their different potentials for differentiation and their various clonal characteristics, including aneuploidy in most cases.

IAP Expression in Embryos

In all mouse strains so far examined, both laboratory and wild, the pattern of morphologically observable IAPs is similar (Yotsuyanagi and Szollosi, 1981). A low number of particles is seen in the maturing stages of oogenesis dropping to undetectable levels in the unfertilized egg and gradually increasing to a burst in abundance by the 2-cell to 8-cell stage. The number of particles at the peak of this burst varies according to strain but in all cases exceeds by orders of magnitude the levels seen at all other stages in fetal or adult tissues. The significance of the dramatic burst of intracisternal particle production for development is completely unknown.

In addition to EM studies of the morphological appearance of IAPs (Calarco and Brown, 1969; Calarco and Szollosi, 1973; Calarco, 1975; Chase and Piko, 1973; Biczysko et al., 1973, 1974; Yotsuyanagi and Szollosi, 1981, 1984; Szollosi and Yotsuyanagi, 1985), several

studies have addressed IAP expression in normal mouse embryos by examination of IAP-related proteins and transcripts. This characterization is, however, incomplete.

Huang and Calarco (1981a) used an anti-IAP antiserum (A'IAP) to immunoprecipitate a set of five proteins from ^{35}S-methionine labeled preimplantation embryos. Proteins of 73 kD, 69 kD, and 67 kD were immunoprecipitated from the 2-cell through 8-cell stage, corresponding to stages of particle presence, while proteins of 75 kD and 77 kD were immunoprecipitated from all stages examined, from the unfertilized egg through blastocyst stages. Using this same antiserum (A'IAP) Huang and Calarco (1981b) examined preimplantation embryos for evidence of cell-surface expression of IAP-related proteins by immunohistochemistry and immunoradiolabeling. They found that this antiserum recognized components of the cell surface of preimplantation embryos from the zygote through eight cell stage, although the surface antigens were not identifed biochemically. Also A'IAP did not detect cell surface antigens on an IAP-producing cell line. The authors speculated that the detected cell surface antigens of the embryos are glycosylated forms of the IAP major core protein, p73.

There has been some discussion in the literature on the possibility that there are two types of intracisternal particles in mouse embryos; a particle abundant at the 2-cell stage designated as "epsilon" and a particle present in much lower numbers in the maturing oocyte and at stages following the 2-cell burst designated

as "large A". The idea that there are actually two distinct types of intracisternal particles in the preimplantation embryo is based on a reported discrepancy between the estimated numbers of IAP-related RNAs and on subtle differences in EM morphology (Yotsuyanagi and Szollosi, 1981, 1984). Piko, Hammons and Taylor (1984) quantified IAP-related transcripts present during oogenesis and preimplantation development of CD2F1 mice (BALB/c x DBA) relative to a IAP-producing myeloma RNA standard by hybridization of restriction fragments of cloned IAP sequences to RNA dot blots. The IAP probes which they used detect sequences in common to both type I and type II sequences. IAP transcript levels in these mice are high in mature oocytes, drop in the unfertilized egg and increase at the late 2-cell stage. Transcript size was determined only for the blastocyst stage where a 5.4 kb transcript was detected. These authors estimate that at the 2-cell stage in these mice the number of IAP-related RNA molecules per embryo is approximately 10,000. Although the number of particles has not been estimated for the particular strain of mice which they examined, they assumed that the number was similar to the maternal Balb/c strain, about 100,000 to 200,000 particles per embryo. Therefore, according to their assumption, there are approximately 10 times as many particles as IAP-related RNA molecules. They concluded that this suggested that the particles which are abundant at the 2-cell stage are genetically distinct from IAPs. But it may not be valid to assume that the F1 hybrids which were examined resemble the maternal strain in numbers of particles produced at the 2-cell stage. Different strains vary in the numbers of particles which they produce. Szollosi and

Yotsuyanagi (1985) have shown that in crosses between low and high producer strains the low producer characteristic is dominant in the offspring regardless of whether it was contributed by the mother or the father. The difference in numbers of particles in low and high producer strains is about 10-fold. So, if the DBA strain parent contributed a low producer character to the offspring, the number of IAP-related RNA molecules might be expected to more closely resemble the number of particles. EM morphometric analysis of the numbers of particles at the 2-cell stage in the CD2F1 mice used by Piko et al. has not been done. In addition it has been shown in the case of other retroviruses that an RNA genome is not mandatory for particle formation (Levin and Rosenak, 1976), so that perhaps it is not unusual that there are more particles than can be accounted for by the number of IAP-RNA molecules present.

On the other hand, immunocytochemical results suggest that these particles are related, if not the same. Antibodies against IAP components (A'IAP) from cell lines clearly recognize the intracisternal particles in EM sections of 2-cell stage embryos (Huang and Calarco, 1982). The resolution of the relationship between the "epsilon" and "large A" particles will have to await the isolation of the particles from embryos and comparison of their RNA genome with that of IAPs.

Transposition of IAP Elements

Interestingly, IAP proviral elements can undergo transposition to new locations within the genome (Hawley et al., 1982, 1984; Rechavi et al., 1982; Lueders et al., 1982; Gattoni-Celli et al., 1983; Sheng-Ong and Cole, 1984; Greenberg et al., 1985; Ymer et al., 1985; Burt et al., 1984), and the transposed IAP sequences can affect the function of genes at the target sites (Table 1). A recent classification scheme (Fanning and Singer, 1987) groups IAP elements with Ty elements of yeast and copia elements of Drosophila as Class I retrotransposons (a term proposed by Boeke et al., 1985). These elements share structural characteristics and mechanisms of reverse transcription. They all possess long terminal repeats (LTRs) which play a critical role in reverse transcription, and each element is associated with a fixed size target site duplication. These features are in contrast to Class II retrotransposons (Drosphila elements F, G and I, the ingi element of Trypanosome brucei, the R2 element of Bombyx mori and the L1 elements of mammals) which do not possess LTRs, are reverse transcribed by an unknown mechanism, and (with the exception of R2, which has no duplications) are associated with variable size target site duplications (Fanning and Singer, 1987).

Although about nine instances of transposition of IAP elements have been reported in the literature, considering the high copy number of IAP proviral elements in the genome it might seem unusual that they do not constitute a more major source of genomic

Table 1. IAP ELEMENT TRANSPOSITIONS (adapted from Kuff and Lueders, 1988)

Target Gene	Cells	Effect on Expression	IAP Element Type	References
x-light chain	igk-20 (MOPC-21)	down	I	Hawley et.al., 1982; Kuff et.al., 1983b Hawley et.al., 1984
x-light chain	igk-1 (MOPC-21)	down	I Δ 1	" " "
interleukin-3	WEHI-3B	up	I Δ 1	Ymer et.al., 1985, 1986
c-mos	XRPC 24	up	I Δ 3	Rechavi et.al., 1982; Kuff et.al., 1983a Canaani et.al., 1983, 1984
c-mos	NSI (MOPC-21)	___	I Δ 3	Gattoni-Celli et.al., 1983; Cohen et.al., 1983
c-myc	J558	___		Greenberg et.al., 1985
renin	germ line DBA/2	up	I Δ 4	Burt et.al., 1984
pseudo a-globin	germ line BALB/C	___	I I(?), I Δ 3	Lueders et.al., 1982
various	MOPC-315	unknown	II B	Sheng-Ong and Cole, 1984

instability. Kuff (1988) has recently summarized a number of factors which may restrict the ability of the IAP sequences to retrotranspose within the genome. These factors concern 1) structural properties of the genetic elements: a high proportion of deleted forms and single-base mutations in open reading frames and primer binding sites, 2) chromosomal effects: localization of elements in constitutive heterochromatin and DNA methylation, 3) properties of the IAPs themselves: unprocessed *gag* and *gag-pol* proteins and their intracisternal location, and 4) cellular supressing mechanisms. Kuff points out that it is interesting that the Ty transposable elements of yeast share many of these restrictive characteristics.

Heterogeneity exists at many levels in the expression of IAP genes. The elements of this heterogeneity are 1) IAP genes themselves, 2) IAP transcripts and 3) IAP proteins. The first of these elements, the complexity of the IAP genes themselves, arises from the fact, as mentioned, that IAP genes are a multigene family of some 1000 members which consist of a variety of full length and deleted forms scattered among most, if not all, chromosomes (Lueders et al., 1977). Secondly, transcripts of IAP genes vary in size according to the genes from which they are transcribed and transcription seems to be influenced by a number of different factors including methylation (Morgan and Huang, 1984, 1987), the presence of transactivating factors (Luria and Horowitz, 1986) and perhaps chromosomal location. Moreover, it has been shown that transcription patterns within the same tissue type can vary in a

strain-related manner (Kuff and Fewell, 1985). Finally, the proteins which are translated from these transcripts can encode a variety of IAP-related proteins; a 73 kD *gag* or core protein (Patterson et al., 1978), a larger 114-120 kD *gag-pol* fusion protein (Kuff et al., 1986), smaller proteins (60 kD) which may act as immunoglobulin binding factors (Moore et al., 1986), and presumably many fragmentary defective proteins. The analysis of IAPs in cell lines has made possible the generation of a number of useful reagents, antisera and cloned DNA probes, which can be employed to study the expression of IAP elements in the preimplantation embryo. The time of IAP expression in the preimplantation embryo is one of dramatic change. With the activation of the embryonic genome and the elimination of the majority of stored maternal RNA at the 2-cell stage (Levey et al., 1978; Piko and Clegg, 1982; Clegg and Piko, 1983; Flach et al., 1982; Young et al., 1978; Sawiki et al., 1982; Bachvarova and De Leon, 1980; Giebelhaus et al., 1983) this period represents the transition from maternal to embryonic control of development (reviewed by Schultz, 1986).

The research which comprises this thesis was undertaken in an effort to examine whether any of these elements of heterogeneity in the expression of IAP sequences correlates with the burst of particle production seen at the 2-cell stage. I have examined the expression of IAP sequences in embryos in three ways. First, I have examined IAP-related proteins by protein blot analysis and by immunoprecipitation using antibodies to components of the intracisternal A particle, primarily the *gag* or core protein.

Secondly, I have examined IAP-related transcripts by RNA slot blot and Northern analysis using type I and type II specific IAP DNA probes. The variability in patterns of IAP transcripts from the thymus of different mouse strains (Kuff and Fewell, 1985) demonstrates that genetic background clearly plays a role in the expression of IAP sequences in this tissue. It seemed possible, therefore, that the IAP sequences expressed during early development could also be subject to a strain-related variability. The best way to answer this question would be to analyze transcripts from embryonic stages of several different strains by RNA blot transfers. Unfortunately this approach was not feasible due to the large number of embryos necessary for RNA preparation. As an alternate approach the expression of IAP sequences was examined in embryonic stem cell lines (ESC) created from four different strains of mice; AKR, C3H, C57/BL and 129. Embryonic stem cell lines are pluripotent cell lines established by culture of the inner cell masses of normal preimplantation mouse embryos in medium conditioned by an established teratocarcinoma stem cell line (Martin, 1981). These cells, by immunological and biochemical properties correspond most closely in developmental capacity to the undifferentiated pluripotent cells in 4- to 6-day embryos (Martin, 1975; Mintz and Illmensee, 1975). After the issue of strain-related variability was examined in this way, the issue of stage-related variability within a single strain was addressed. IAP transcripts from the 2-cell through blastocyst stage embryos of the ICR strain were examined with type specific IAP DNA probes on RNA slot blots.

Finally, I have examined type I and type II IAP transcripts by amplification of embryo cDNAs using specific oligonucleotide primers in the polymerase chain reaction (Saiki et al., 1985, 1988). This technique is a primer-directed enzymatic amplification of specific DNA sequences. Primers which bracket the sequence of interest are synthesized and used to anneal to complemetary strands of the DNA. A thermostable polymerase (from *Thermus aquaticus*) extends the DNA by synthesis and the two strands of the product are separated by heat denaturation in preparation for another round of annealing and extension. The entire cycle is repeated many times resulting in a many-fold amplification of the particular sequence of interest. This approach has been used to demonstrate the presence of mouse embryonic transcripts for growth factors and growth factor receptors (Rappolee et al., 1988), proto-oncogenes (Nielsen et al., 1988) and metalloproteinases (Brenner et al., 1988).

Two types of IAP genes have been distinguished: Type I and Type II. Type I genes are considered to be full length (7 kb) while Type II genes are characterized by deletions of a significant portion of the 5' sequences. In addition to these deletions, type II IAP genes contain a unique insertion of approximately 270 base pairs (Fig. 1). DNA oligonucleotide primers (AP1, AP2, and AP3) were designed which, when used in PCR, act to amplify IAP sequences within cDNAs synthesized from the RNA of preimplantation mouse embryos. RNA is extracted from embryos and is then copied into a single stranded cDNA by reverse transcription. This cDNA is then used as the

template for amplification by PCR. These amplified sequences can be distinguished as derived from type I or type II IAP transcripts on the basis of their size as evaluated on ethidium bromide stained agarose gels. This strategy does not allow a distinction to be made about the subtypes of either type I or type II IAP sequences. The identity of the amplified sequences is confirmed by hybridization of Southern transfers to type I and type II specific IAP DNA probes. As a control for genomic DNA contamination of the RNA samples, an aliquot of DNAse treated RNA is set aside, subjected to alkaline hydrolysis to remove the RNA, neutralized and then amplified by PCR.

The strategy which was used to design primers for the amplification was to choose sequences which were unique to either type I or type II sequences for the downstream primers and a primer complementary to a sequence common to both types I and II for the upstream primer. The PCR products that this strategy predicts are a 417 base pair sequence for amplification of type I IAP cDNAs and two sequences of 242 and 292 base pairs for amplification of type II IAP cDNAs (Fig. 3).

Fig. 2. Schematic illustration of the experimental design for embryo cDNA preparation and PCR. Samples of 100-200 embryos were lysed in guanidine thiocyanate and beta-mercaptoethanol. *E. coli* rRNA was added as carrier and the mixture was pelleted through a cushion of cesium chloride. The nucleic acid pellet was resuspended, precipitated with ethanol and then resuspended once more and digested with an RNAse-free DNAse. The RNA was then copied into cDNA by reverse transcriptase. This cDNA was then used as the template in the PCR. As a control for genomic DNA contamination of the RNA samples, an aliquot of DNAse-treated RNA was set aside, subjected to an alkaline hydrolysis, neutralized and the run through the PCR.

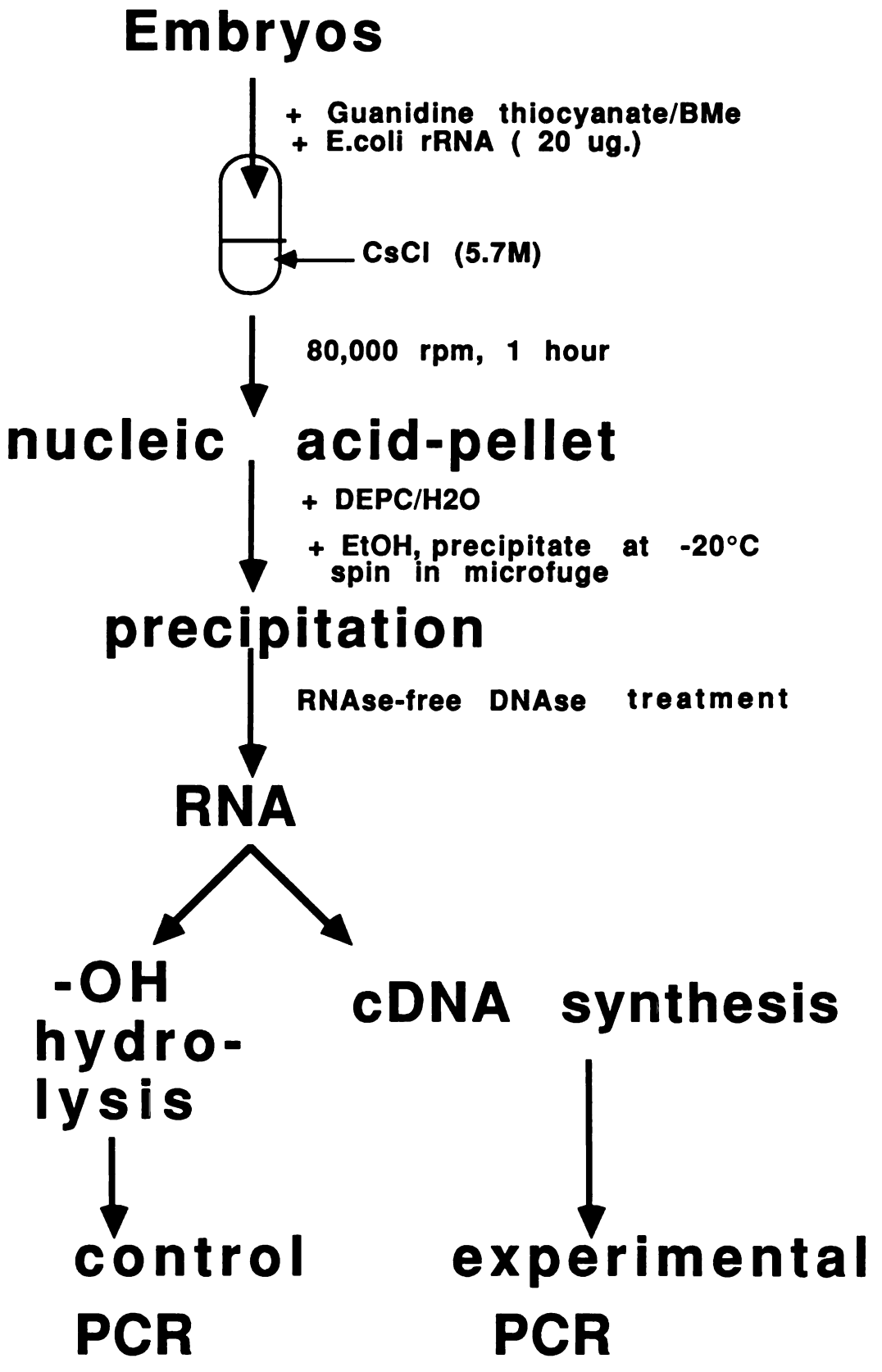
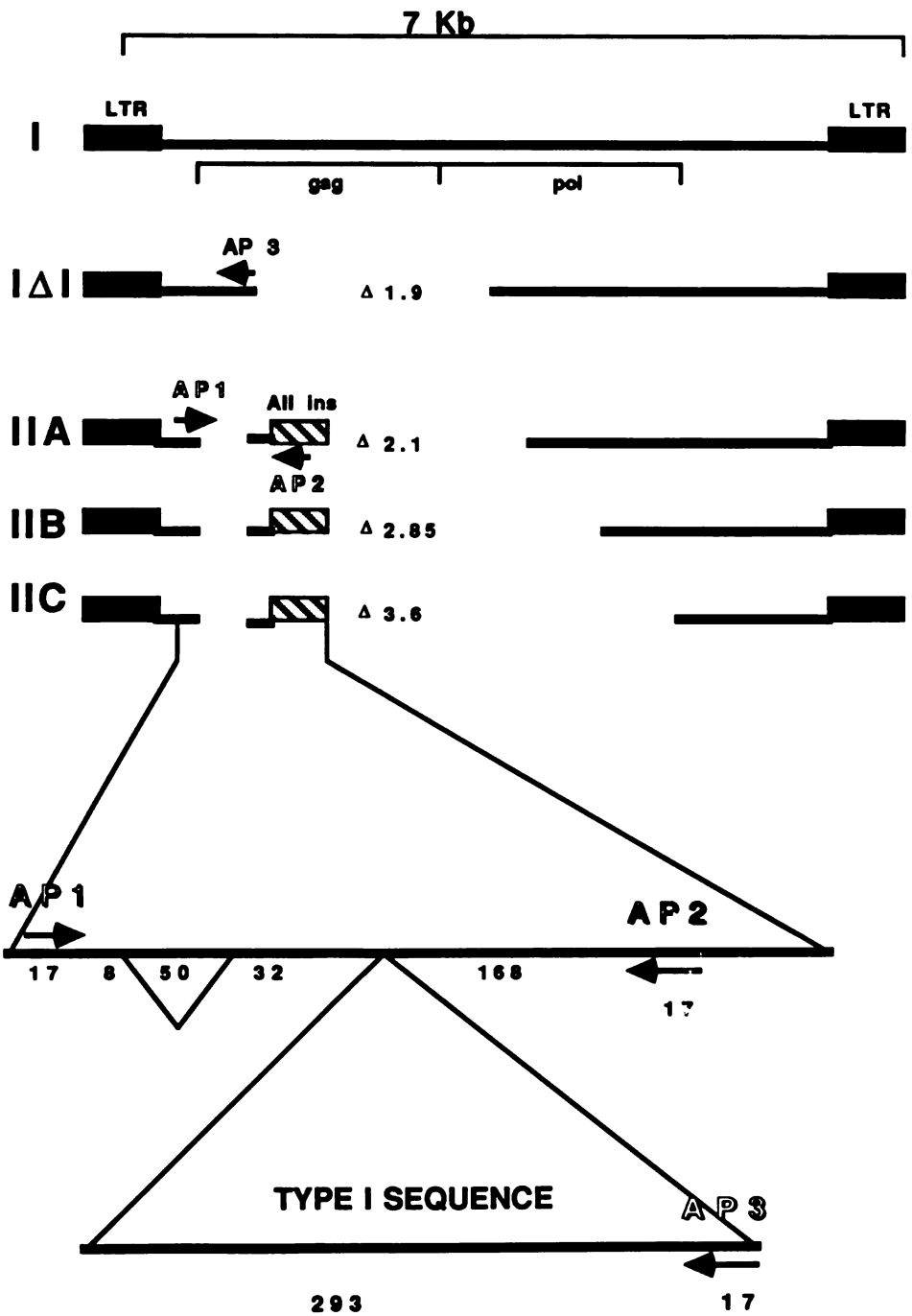

Embryos
+ Guanidine thiocyanate/BMe
+ E.coli rRNA (20 ug.)
CsCl (5.7M)
80,000 rpm, 1 hour
nucleic acid-pellet
+ DEPC/H2O
+ EtOH, precipitate at -20°C
spin in microfuge
precipitation
RNAse-free DNAse treatment
RNA
-OH hydro-lysis
cDNA synthesis
control PCR
experimental PCR

Fig. 3. Polymerase chain reaction primer design. Sequences unique to type I (AP3) and type II (AP2) IAP sequences were used as the downstream primers. A sequence common to type I and type II sequences was used as the upstream primer (AP1). All primers are 17 nucleotides in length. The amplification products predicted by this strategy are 417 bp for type I sequences and 240 bp and 292 bp for type II sequences (50 bp are deleted in some type II sequences resulting in the 240 bp amplification product). Primer AP2 is located within the type II specific AIIins sequence (indicated by the hatched box).



TYPE I PRODUCT = 417 BP

TYPE II PRODUCT = 242 AND 292 BP

MATERIALS AND METHODS

Embryo Culture

Female ICR mice (12 weeks old, Harlin) are superovulated by injection with 5 IU of pregnant mare's serum (Teikoku Hormone Mfg. Co., Japan) followed after 48 hours by an injection of 2.5 IU of human chorionic gonadotropin (hCG, Sigma). The mice are then caged with ICR males, and inspected the next morning for the presence of a vaginal plug, indicating insemination. Embryos are collected at the 2-cell stage (48 hours post-hCG) by flushing them from the oviducts using modified embryo culture medium (Biggers et al., 1971; Spindle, 1980), and then cultured in microdrops (15-20 ul) to obtain the desired stages. Incubation of culture drops takes place under a layer of mineral oil, at 37°C, in a humidified atmosphere of 5% CO_2 in air. Unfertilized eggs are obtained from superovulated but unmated ICR females and follicle cells are removed by a brief incubation in hyaluronidase (75 units/ml Sigma).

Antisera

Anti-IAP (A'IAP) is a polyclonal rabbit antiserum made against a preparation of myeloma (MOPC 104-E) particles that had been treated with deoxycholate to remove the outer shell leaving primarily the cores (provided by Dr. Nelson Wivel, NCI) (Huang and Calarco, 1981). Before use the serum was heat inactivated (56°C, 30 minutes) and absorbed three times with crude membrane homogenates of adult mouse livers, kidneys, and spleens. IgG fractions were prepared by precipitation of the serum with 40%

saturated ammonium sulphate followed by separation of DEAE-Sephadex A-50 (Pharmacia) (Fahey and Terry, 1973). Normal rabbit serum and IgG fractions were prepared as above from non-immune rabbits.

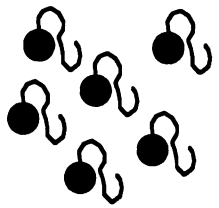
Anti-p73 (A1.3), a polyclonal rabbit antiserum made against electrophoretically purified IAP *gag* core protein (p73) (Kuff et al.,1980), was prepared by repeated injections of emulsified gel slices containing p73, and was a gift of Drs. Kuff and Lueders, NCI.

Anti-peptide antisera: anti-6016, anti-6020 and anti-6021, are polyclonal rabbit antisera made against ovalbumin-conjugated synthetic peptides (BIOSEARCH) whose amino acid sequences were derived from a cDNA clone (8.3) of an IAP element (Moore et al., 1986) and were gifts of Kevin Moore, DNAX. The synthetic peptides were conjugated to ovalbumin using the reagent of Rector et al. (Rector et al.,1978). Rabbits were immunized with 500 ug of conjugated peptide in complete Freund's adjuvant, boosted 4 weeks later with 500 ug of conjugated peptide in incomplete Freund's adjuvant, and bled 1 week after the boost. Peptide 6016 is: (Cys) Glu Thr Val Lys Ala Ala Leu Pro Ser Met Gly Lys Tyr Met. Peptide 6020 is: (Cys) Gly Arg Val His Ala Pro Val Glu Tyr Leu Gln Ile Lys Glu Ile, nucleotides 772 to 816 of the IAP/IgEBF clone 8.3 sequence. Peptide 6021 is: (Cys) Gly Glu Gly Gln Phe Ala Asp Trp Pro Gln Gly Ser Arg Leu Gln, nucleotides 572 to 606 of the 8.3 sequence. The amino acid sequences correspond to portions of the *gag* region (Fig. 4).

Fig. 4. Antisera preparations. The antigens used to generate the three types of anti-IAP antisera are shown. A'IAP was generated against a dexycholate-treated preparation of IAPs from a myeloma cell line (MOPC 104E) (Huang and Calarco, 1981). A'p73 was generated against a gel purified 73 kD IAP core protein (Kuff et al., 1980). A'6016, A'6020 and A'6021 were generated against synthetic peptides whose sequences were derived from the *gag* coding region of a cloned IAP gene (Moore et al., 1986).

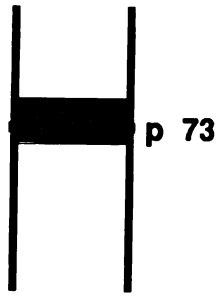
Antisera

particle cores



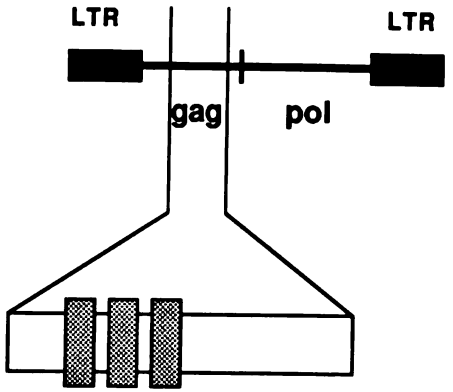
A'IAP

gel slice



A'p73

peptides



A'6016
A'6020
A'6021

Protein Blotting

The protein blotting procedure was adapted from Towbin et al. (1979). Embryos are rinsed through three changes of culture medium containing 3 mg/ml polyvinylpyrolidine in place of BSA. The embryos are lysed by the addition of 20 ul of lysing buffer (1% NP-40, 0.4% sodium deoxycholate, 30 mM Tris/HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 1.5 M NaCl, 0.5 mM PMSF) and incubated on ice for 5 minutes. The lysate is denatured by heating at 100°C for 3 minutes after the addition of SDS to a final concentration of 0.5% and dithiothreitol (DTT) to 1 mM. After centrifugation in a microfuge for 5 minutes at 4°C to remove insoluble material, iodoacetimide is added to the supernatant to a final concentration of 5 mM in order to eliminate excess DTT and to alkylate proteins.

Embryo lysate is separated on a 7.5% SDS-PAGE gel which is then equilibrated in transfer buffer (2 mM sodium acetate, 5 mM MOPS pH7.5 in 20% EtOH) for 30 minutes. Nitrocellulose is cut to the size of the gel, soaked in transfer buffer and a sandwich is assembled consisting from bottom to top of the transfer grid, scotch pad, 2 sheets of #1 Whatman filter paper, gel, nitrocellulose, 2 sheets of #1 Whatman filter paper, transfer grid. All components of the sandwich are prewet with transfer buffer and assembly occurs partially submerged taking care to eliminate any air bubbles between layers. The sandwich is lowered into the electrotransfer chamber which is filled with buffer. The transfer apparatus is run at 200 volts for 2

hours with a cooling coil or overnight at 30 volts with a cooling coil, and a final 1 hour at 100 volts.

Transferred proteins are visualized by staining in Ponceau S solution (0.5% Ponceau S, 1% glacial acetic acid in water) for 5 minutes and destained in water for 3 minutes (Salinovich and Montelaro, 1986). Unbound sites on the nitrocellulose are blocked by incubation in BLOTTO (Johnson et al., 1984, 5% non-fat dry milk, 0.01% antifoam A, 0.01% thimerisol in PBS) and 5% normal goat serum overnight at 4°C. The blot is incubated in primary antibody diluted in the BLOTTO (1:100) for 1 hour at room temperature on a shaking platform and washed 5 times for 3 minutes each with 0.1% Tween-20 in PBS. The blot is then incubated in secondary antibody diluted in BLOTTO (1:500) at room temperature for 1 hour.

The secondary antibody is conjugated either to biotin or to horseradish-peroxidase. Following incubation with a biotinylated secondary antibody, the blot is washed 5 times for 3 minutes each with 0.1% Tween-20 in PBS and then incubated in an avidin-conjugated alkaline phosphatase (Vector) diluted in BLOTTO (1:500) for 30 minutes. The blot is washed 5 times and transfered to a phosphatase substrate solution (Vectastain ABC-AP) and allowed to develop for 30 minutes. The blot is then rinsed in water and photographed. If a horseradish-peroxidase conjugated secondary antibody is used, the blot is washed 5 times for 3 minutes each with 0.1% Tween-20 in PBS and then developed in substrate solution (0.05% 3,3'-diaminobenzadine in PBS, 0.02% cobalt chloride, 0.03%

H_2O_2). The reaction is terminated by rinsing the blot in water several times. The blot is then photographed.

Immunoprecipitation of Radiolabeled Embryos

Approximately 100 embryos are washed with methionine-free flushing medium and placed under mineral oil in a 10 ul drop of methionine-free medium containing 100 uCi of ^{35}S-methionine (specific activity, 1mCi/mmol, Amersham). After a 4 hour incubation at 37°C in an atmosphere of 5% CO_2, the embryos are washed 4 times in methionine-free medium.

The preparation of extracts is a modification of Kuff and Fewell (1985). Labeled embryos are collected, washed and then lysed by suspension in 100 ul of lysing buffer (1% NP-40, 0.4% sodium deoxycholate, 30 mM Tris/HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 1.5 M NaCl, 0.5 mM PMSF). This mixture is incubated on ice for 5 minutes. The lysate is denatured by heating at 100°C for 3 minutes after the addition of SDS to a final concentration of 0.5% and DTT to a final concentration of 1 mM. The lysate is then spun in a microfuge for 5 minutes at 4°C to remove insoluble material. Iodoacetimide is added to the supernatant to a final concentration of 5 mM in order to eliminate excess DTT and to alkylate proteins. A 2ul aliquot is removed from the sample for determination of the radioactivity incorporated into trichloroacetic acid-precipitable material.

Each sample of embryo lysate is preabsorbed for 15 minutes on ice with 10 ul of normal rabbit serum (final concentration 1 mg/ml). Precipitates are collected on a 100 ul pellet of Protein A Sepharose beads (Pharmacia). After brief centrifugation to pellet the beads, the supernatant is removed to a new tube and the pellet is discarded. The preabsorbed lysate is divided into two equal volumes. One volume is incubated with 10 ul of primary antiserum or IgG (final concentration 0.5 ug/ml) and the other volume is incubated with 10 ul of normal rabbit IgG or serum. The samples are incubated at 4°C overnight on a rocking platform. The samples are then added to 100 ul pellets of Protein A Sepharose beads and rocked for 1 hour at 4°C. After a brief centrifugation the supernatants are discarded and the pellets are washed twice with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1M sodium phosphate pH 7.2, 10 mM EDTA) and twice with a solution containing 0.05% NP-40, 0.15 M NaCl, 50 mM Tris/HCl pH 7.4, and 5 mM EDTA. The immunoprecipitated proteins are eluted with 40 ul of SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Polyacrylamide Gel Electrophoresis

Immunoprecipitates are analyzed following the method of Laemmli (1970) using 7.5% polyacrylamide slab gels containing SDS. Prestained molecular weight markers (BRL) are used, and consist of myosin (200 kD), phosphorylase b (97.4 kD), bovine serum albumin (68 kD) and ovalbumin (43 kD). The gels are stained with Coomassie

Brilliant Blue, fixed, enhanced (ENHANCE, New England Nuclear), dried down, and exposed to x-ray film (Kodak XAR-5) at -80°C.

Embryonic Stem Cell RNA Blot

An RNA transfer blot of embryonic stem cell lines (ESC) created from four different strains of mice (AKR, C3H, C57/BL and 129) was a generous gift of Thiennu Vu, University of California, San Francisco. Each lane represents 20 ug. of poly A+ RNA.

RNA Slot Blotting

The procedure of RNA slot blotting was adapted from Piko et al. (1983). Batches of embryos are collected and stored at -80°C in the presence of RNAse inhibitor (vanadyl ribonucleosidase inhibitor, BRL) until use. Embryo samples are digested with 0.5% SDS containing 100 ug/ml proteinase K and total RNA is extracted in the presence of E. coli tRNA as carrier once with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform/isoamyl alcohol (24:1). The RNA is precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of EtOH at -20°C overnight. The RNA is spun in a microfuge for 15 minutes at 4°C and the pellet is rinsed in 70% EtOH and dried in a spinvac. The RNA is digested with DNAse at 37°C for 15 minutes. RNA is denatured by heating in 0.02 M sodium phosphate pH 7, 1 mM EDTA, 2.2 M formaldehyde, 50% formamide (deionized) at 60°C for 5 minutes. The denatured RNA is then brought to 20 X standard

saline citrate (1X is 0.15 M NaCl, 0.015M sodium citrate) in a volume of 100-200 ul. and dotted onto a nitrocellulose sheet. The RNA is baked onto the membrane at 80 °C for 2 hours.

The blot is prehybridized for 3 hours at 42°C in 1 M NaCl, 50 mM sodium phosphate pH 7, 0.02% BSA-Ficoll-PVP, 0.1% SDS, 4 mM EDTA, 50% formamide and 100 ug of sonicated denatured calf thymus DNA/ml The blot is then hybridized with the same buffer with 1 X 107 cpm/ml of ^{32}P-labeled DNA probe for 20 hours at 42°C. Following this the blot is then washed 3 times for 5 minutes at 25°C and 6 times for 20 minutes at 68°C in 0.25 M NaCl, 12.5 mM sodium phosphate pH 7, 1 mM EDTA and 0.1% SDS. Finally, the blot is dried and autoradiographed for 1-4 days at -70°C on preflashed film with an intensifying screen.

Growth and Isolation of Plasmid DNA

I used a modification of a technique of Birnboim and Doly (1979) as communicated by Mike Frohman, UCSF. A 40 ml culture of *E. coli* is grown in a 500 ml Erleneyer flask to O.D.> 1.5 (overnight, not >24 hours) with appropriate antibiotics in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl). The culture is then transfered to 15 ml polypropylene (Falcon, #2059) tubes and spun in the centifuge for 5 minutes at 5000 r.p.m.. The supernatant is discarded and the pellets are resuspended in 1 ml of GTE (50 mM glucose, 25mM Tris-Cl pH 8, 10 mM EDTA pH 8). After vortexing, 2 ml of NS (200 mM NaOH, 1% SDS) are added and the suspension is mixed gently by inversion until

homogeneous. One ml of 5M potassium acetate (3M potassium acetate, 2M acetic acid) is then added and once again the suspension is mixed gently by inversion until homogeneous. At this point the product resembles egg drop soup. After centrifugation for 8 minutes at 7500 rpm, the supernatant is transfered to a fresh tube using a pipet. 3 ml of isopropanol is added, the solution is mixed and spun in the centrifuge for 5 minutes at 5000 r.p.m. . The supernatant is completely removed and the pellet is resuspended in 500 ul. of 50 mM Tris (pH 8.3) and transfered to a microfuge tube. After the addition of 250 ul. of 7.5 M ammonium acetate the solution is mixed well. The solution is frozen for 3 minutes in a dry ice/ethanol bath, and then immediately spun in the centrifuge for 2 minutes. Again the supernatant is completely removed and the pellet is rinsed with 70% ethanol, dried, and resuspended in 100 ul. of TE (10 mM Tris-Cl, 1 mM EDTA). The yield is approximately 100 ug. of DNA.

Preparation of Radioactive Probe

For the detection of both type I and type II sequences, an IAP recombinant plasmid pMIA1 (Lueders and Kuff, 1980) was used as a probe and prepared in general according to instructions of the manufacturer, BRL. It contains 5.2 kb of internal sequence (see Fig. 1) from a genomic IAP sequence inserted into the HindIII/EcoR1 site of pBR322 and was a gift from Kira Lueders and Ed Kuff, NCI . For the detection of type I sequences, a type I specific probe was used (Wujcik et al., 1984). This probe contains a 1.0 kb SstI to Bam H1 sequence from a cloned IAP gene (IAP 81), inserted into M13 mp18

and was a gift of Ru Chih Huang, Johns Hopkins. For the type II IAP probe I used a recombinant plasmid pTAII.1, containing 3 copies of a 303 bp. Hae III fragment carrying the type II IAP element specific insertion AIIins (see Fig. 1) in the pT7/T3-19 vector (BRL) which was a gift from Kira Lueders, NCI. The type II specific sequence used to derive this probe was originally cloned by Sheng-Ong and Cole (1984). The alpha-actin cDNA probe (a gift from Randy Moon, University of Washington, Seattle) was originally cloned by Minty et al. (1981). It consists of a 1350 bp. nucleotide sequence inserted into the pstI site of pBR322 using the poly(dC)-poly(dG) tailing procedure. The insert represents approximately 90% of the coding sequence for mouse alpha-actin plus about 300 nucleotides of non-coding sequence. The coding sequence cross-hybridizes completely with non-muscle actin mRNAs (Minty et al., 1981). The plasmids are labeled by nick translation (BRL kit) to a specific activity of 2 X 10^8 cpm/ug of DNA, using (alpha-^{32}P) dCTP (6000 Ci/mmole, NEN) in a reaction containing 200 ng of probe DNA.

Densitometry

The autoradiographs of the RNA slot blots were scanned with a laser-densitometer. The ratio of the amount of beta-actin RNA per embryo at the 8-cell stage to the blastocyst stage is 1 to 13.3 (derived from Giebelhaus et al., 1985). From the ratio of the densitometric signals from the 8-cell and blastocyst samples on the autoradiographs of the slot blots hybridized with the actin probe, the ratio of embryo RNA equivalents between these stages was deduced.

This factor was used to normalize the densitometric signals from the same slot blots hybridized to the IAP probes and these values were used to compare relative transcription of type I and type II IAP sequences at these stages.

Southern Blotting

The technique which I used is a modification of that of Southern (1975), communicated by Mike Frohman, UCSF. DNA samples are run using 2% agarose gels and TAE buffer (40 mM Tris-acetate, 2 mM EDTA pH 8) containing 0.5 ug/ml. ethidium bromide. Loading buffer contains both bromphenol blue and xylene cyanol. Samples are electrophoresed at 150 volts in a minigel box. The gel is photographed on short wave length UV and then exposed to UV for 30 seconds to nick the DNA. The gel is then denatured (1.5 M NaCl, 0.5M NaOH) until the xylene cyanol turns green (about 5 minutes) using a shaking platform. If no xylene cyanol is present denaturation proceeds for 20 minutes.

The gel is transfered to prewet Genescreen membrane (NEN) in denaturation solution (1.5 M NaCl, 0.5M NaOH) by capillary action. Transfer is allowed to proceed overnight. The blot is marked on the back and floated (DNA side up) on neutralizing solution (1M Tris/HCl pH 8, 1.5M NaCl) until the xylene cyanol turns blue (less than 1 minute). The blot is swished to submerge and soaked for 1 minute. The blot is then rinsed in 2X SSC (0.3 M NaCl, 0.03 M sodium citrate pH 7) and, while wet, wrapped in saran wrap. The DNA fragments

are then covalently bound to the membrane by UV crosslinking, achieved by exposure to a 6-15 W germicidal UV lamp at a distance of 25 cm for 2.5 minutes (Church and Gilbert, 1984).

Hybridization of Southern Blots

Hybridization of Southern blots was performed according to Church and Gilbert (1984). Blots are prehybridized in Church buffer (1% bovine serum albumin, 0.4 M sodium phosphate pH 7.2*, 1 mM EDTA pH 8, 7% SDS pH 7.2, 15% deionized formamide) at 65°C for 5 minutes and hybridized in the same buffer with a radioactively labeled probe of 10^7 cpm/ml (specific activity > = 2 X 10^8 cpm/ug.) or greater (not exceeding 5 X 10^7 cpm/ml.) at 65°C for 1 hour. The blots are washed at low stringency for 5 minutes (2X SSC, 1% SDS, 50°C), at medium stringency for 10 minutes (0.2X SSC, 0.1% SDS, 50°C), and at high stringency for 15 minutes (0.2X SSC, 0.1% SDS, 65°C). The blots are then wrapped in saran wrap and exposed to film for 5 minutes to several hours and developed.

* (1M sodium phosphate at pH 7.2 is made by dissolving 134 g of $Na_2HPO_4 \cdot 7H_20$ and 4 ml of 85% H_3PO_4 per liter)

RNA Isolation

The technique of RNA isolation is a microadaptation of the method of Chirgwin et al. (1979) by Carol Brenner and Richard Schultz, as used by Rappolee et al. (1988). Samples of at least 100 embryos are rinsed in culture medium and then spun down in a

RNAse-free microfuge tube (treated with 0.1% diethylpyrocarbonate (DEPC) in water overnight, and then autoclaved). Excess medium is drawn off and 100 ul of 4M guanidine thiocyanate, 1% beta-mercaptoethanol is added as a chaotropic agent. *Escherichia coli* rRNA is added in the amount of 10 ul (20 ug) per sample as carrier. (At this point the sample is frozen at -80°C if it is not to be immediately used). The embryo extract is layered onto a cushion of 100 ul. of 5.7 M cesium chloride (CsCl) and spun in an ultramicrocentrifuge (Beckman TL-100) at 80,000 rpm. for 1 hour at 20°C. The liquid phase is drawn off and the RNA pellet is resuspended in 50 ul of DEPC-treated H_20. A 1/10 volume of 3 M sodium acetate (NaOAc) and a 2.5 volume of EtOH is added and the RNA is allowed to precipitate at -20°C overnight. The RNA precipitate is pelleted by spinning in a microfuge for 15 minutes at 4°C. The pellet is washed twice in cold 80% EtOH and then dried in a speedvac.

DNAse Treatment

DNAse treatment is performed according to instructions of the manufacturer, Promega Biotec. The RNA is taken up in RQ1-DNAse buffer (40 mM Tris/HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$) and digested with 2 ul (2 units) of RQ1-DNAse (Promega Biotec) for 2 hours at 37°C. After digestion the samples are extracted once with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform/isoamyl alcohol (24:1). The RNA is precipitated at -20°C overnight after the addition of a 1/10 volume of 3M NaOAc and a 2.5

volume of EtOH. The RNA precipitate is pelleted in a microfuge for 15 minutes at 4°C. The pellet is washed twice in cold 80% EtOH, dried in a speedvac and then resuspended in 20 ul of DEPC-treated dH_20.

First Strand cDNA Synthesis

The first strand synthesis was a modification of that in Maniatis et al. (1982), communicated by Mike Frohman, UCSF. A mixture is assembled on ice consisting of reverse transcriptase buffer (50 mM Tris/HCl pH 8.5, 40 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 15 mM dNTPs (Pharmacia)), oligo-deoxythymidine (0.5 ug) and vanadyl ribonucleosidase inhibitor complex (10 units). The DNAsed-RNA is added to this mixture immediately after its resuspension in DEPC-treated H_2O from the ethanol precipitate (a 2 ul aliquot of RNA is reserved in another tube to serve as a control for PCR). Reverse transcriptase from Avian myeloblastosis virus (Life Sciences, Inc.) is added (0.9 ul.=23 units) and the mixture is allowed to sit at room temperature for 5 minutes (this is thought to facilitate annealing of oligo-dT to poly-dA tracts). The reaction is then incubated at 42°C for 2 hours. The reaction is stopped by the addition of 80 ul. of TE (10 mM Tris-Cl, 1 mM EDTA).

Amplification of Nucleic Acid Sequences or Polymerase Chain Reaction (PCR)

The PCR was performed according to Saiki et al. (1985, 1988), modified by Michael Frohman, UCSF. Oligonucleotide primers (OPERON, San Pablo, CA) were delivered as lyophilized samples of 0.2 micromoles each. Each sample was taken up in plasmid-free water. The exact concentration was calculated by checking the optical density at 260 wavelength with a spectrophotometer (OD 1= 35 ug/ml). Aliquots of each sample were then diluted to a working concentration of 150 ng/ul. and frozen at -80°C. The sequence of the primers is as follows:

AP1 5'-AGC AGG TGA AGC CAC TG-3'
AP2 5'-CAA TCC CTC TGC AGC TC-3'
AP3 5'-CTT GCC ACA CTT AGA GC-3'

Primer AP1 corresponds to nucleotide positions 1552-1568 in MIA14 (Mietz et al., 1987), a full length IAP gene which has been completely sequenced. Primer AP3 corresponds to nucleotide positons 2050-2066 in MIA14. Primer AP2 corresponds to nucleotide positions 164-180 within the type II specific AII ins sequence in the cloned IAP gene II3 (Lueders and Mietz, 1986) (see Fig. 1).

Beta-actin primers were generously provided by Dan Rappolee, University of California, San Francisco:

5' oligonucleotide 5'-C GTG GGC CGC TCT AGG CAC CA-3'
3' oligonucleotide 5'-T TGG CCT TAG GGT TCA GGG GGG-3'

These primers span an exon/intron border of the beta-actin gene. The PCR product of amplification of the cDNA is 243 bp. and the PCR product of amplification of the genomic DNA is 373 bp, larger by the size of the 130 bp. intron sequence.

Embryo cDNA (5 ul) is combined with a mixture containing *Thermus aquaticus* (Taq) DNA polymerase buffer (670 mM Tris/HCl pH 8.8, 67 mM $MgCl_2$, 1.7 mg/ml BSA, 166 mM $(NH_4)_2SO_4$) (Frohman et al., 1988), deoxynucleotide triphosphates (dCTP, dGTP, dATP,and dTTP at 15 mM each, Pharmacia), DMSO, and the 5' and 3' oligonucleotide primers (25 pmol each) in a total volume of 52 ul. in a 0.6 ml. double snap locking tube (Robbins Scientific, Mountainview). The tube is placed into a thermocycler machine (Perkin Elmers-Cetus) which is programmed as follows. The mixture is heated for 5 minutes at 95°C (this initial denaturation is primarily to kill potential proteases which may be left over from DNA preparation). The mixture is then cooled to 72°C and kept at this temperature while polymerase is added (one prevents misannealing of primers by keeping reaction mixture at or above annealing temperature at all times). Taq polymerase (0.5 ul) is added (Perkin Elmers-Cetus; 2.5 units/ul.). The mixture is overlaid with 30 ul. of

mineral oil (Sigma #400-5) which is at 72°C and the lid of the tube is snapped tightly shut. Control tubes containing all components of the reaction except the cDNA template are included in each PCR run. This control is important in the detection of false positive amplifications which can use contaminating DNA introduced by aerosols at some point in the preparations.

The amplification program consists of three steps; denaturation, annealing, and extension. These three steps constitute 1 cycle. The amplification program repeats for 40 cycles. Denaturation proceeds at 94°C for 40 seconds, annealing proceeds at 50°C for 1 minute and extension proceeds for 1 minute at 72°C. A final extension step is carried out, after the 40 cycles, at 72°C for 15 minutes to promote fully extended DNA. The PCR products are analyzed on 2% agarose gels containing ethidium bromide.

RESULTS

Protein Blot

Possibly the most sensitive method for detecting antigens on Western blots relies on the interaction of a biotinylated secondary antibody with a streptavidin-conjugated enzyme (Oberfelder, 1989). In the final step of this procedure the enzyme is reacted with a substrate producing a visible precipitate. The high degree of sensitivity (1 ng) of this technique makes this a method of choice for embryo samples where a limited amount of material is available. A less sensitive technique (to 50 ng) which employs a secondary antibody directly conjugated to horse-radish peroxidase was also used.

The protein blot analysis using both detection systems shows that several different anti-IAP antisera recognize the 73 kD IAP core protein from the unfertilized egg through 8-cell stage (Figs. 5, 6 and 7). The anti-peptide antisera, anti-6020 and anti-6021, appear to be the best at detecting this protein. All antisera have in common the recognition of p73, but additional proteins are also recognized by the different antisera.

In all samples examined, the 73 kD protein is recognized prominently by the anti-6020 and anti-6021 antisera and less so but

Fig. 5. Immunodetection of IAP-related proteins on protein blots of mouse unfertilized eggs and embryos by various anti-IAP antisera. IAP-related proteins of unfertilized eggs (UE) and zygotes (Z) were detected with anti-6021 and A'IAP antisera. IAP-related proteins of 2-cell embryos were detected with anti-6016, anti-6020, anti-6021 and A'IAP antisera. IAP-related proteins of 8-cell embryos were detected with anti-6020 and anti-6021 antisera. The 73 kD IAP *gag*-related protein is recognized in all stages examined. Additional proteins are recognized by the various anti-IAP antisera: 60 kD (anti-6020), 100 kD and 83 kD (A'IAP). Non-specific proteins (160 kD and 90 kD) are detected by the biotin/streptavidin based detection system regardless of the primary antisera which is used. A normal rabbit serum (NRS) control immunoreaction is shown for the UE and 2-cell stages. NRS controls for the other stages were similarly negative. Molecular weight size markers are myosin (200 kD), phosphorylase b (97.4 kD) and bovine serum albumin (68 kD).

Western

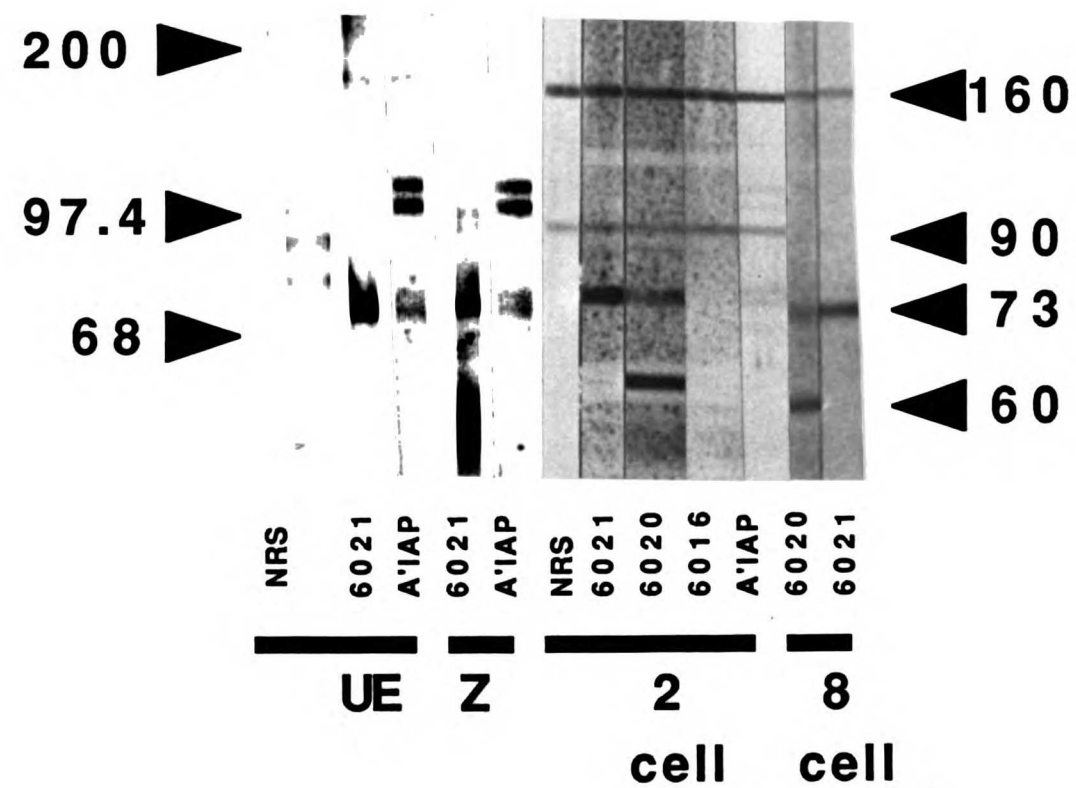

200
97.4
68
160
90
73
60
NRS
6021
A'IAP
6021
A'IAP
NRS
6021
6020
6016
A'IAP
6020
6021
UE
Z
2
cell
8
cell

Fig. 6. Comparison of immunodetection systems on protein blots of 8-cell mouse embryos. Anti-6016, anti-6020, anti-6021, A'IAP and normal rabbit serum (NRS) were used with either a biotin/streptavidin based detection system (ABC-AP) or with a secondary antibody directly conjugated to horse radish peroxidase (GAR-HRP). The 73 kD IAP *gag*-related protein is recognized by antisera using both secondary detection systems, as are the additional proteins recognized by specific anti-IAP antisera: 60 kD (anti-6020), 43 kD (anti-6021). Non-specific proteins (160 kD and 90 kD) are detected by the biotin/streptavidin based detection system (ABC-AP) regardless of the primary antisera which is used. These proteins are not detected when a goat-anti-rabbit antisera conjugated to horseradish peroxidase (GAR-HRP) is used as the secondary detection system. Molecular weight size markers are myosin (200 kD), phosphorylase b (97.4 kD), bovine serum albumin (68 kD) and ovalbumin (43 kD).

Western
8 cell

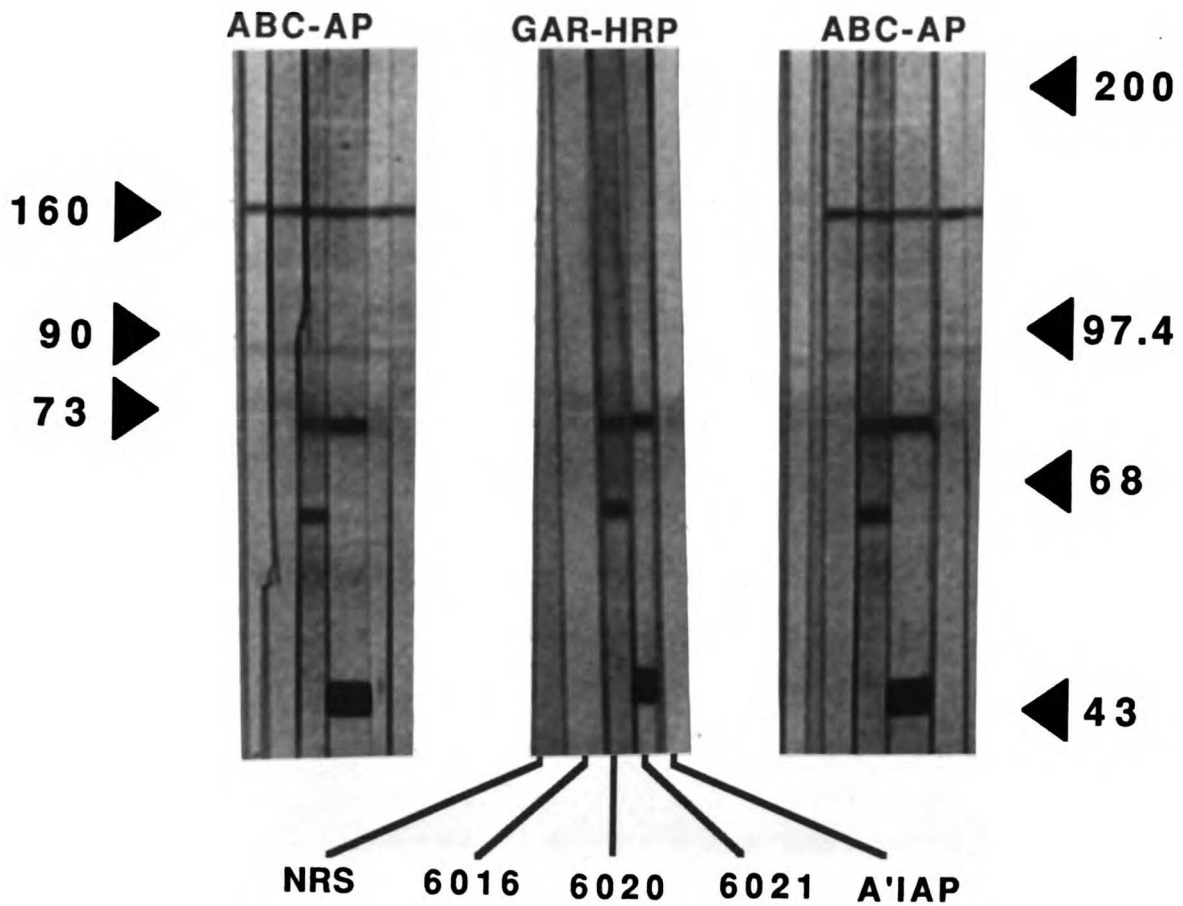
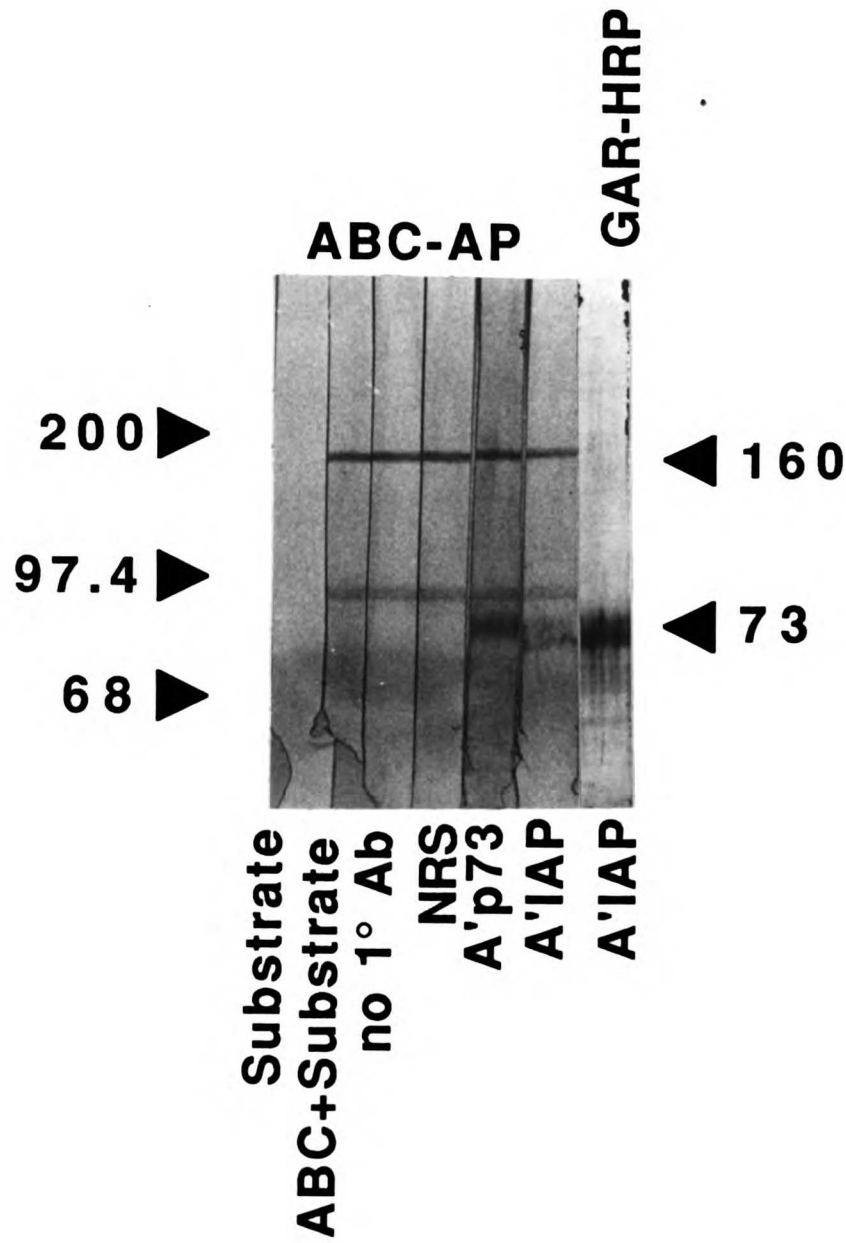


Fig. 7. Detection of non-specific proteins on protein blots of 8-cell mouse embryos using a biotin/streptavidin based detection system (ABC-AP). The 73 kD IAP *gag*-related protein is recognized by the anti-IAP antisera (A'p73 and A'IAP). Non-specific proteins (160 kD and 90 kD) are detected by the biotin/streptavidin based detection system (ABC-AP) regardless of the primary antisera (A'IAP, A'p73 or NRS) which is used, or whether any primary antisera is used at all ("ABC+substrate", "no 1° Ab" lanes). These proteins are not detected when a goat-anti-rabbit antisera conjugated to horseradish peroxidase (GAR-HRP) is used as the secondary detection system. Molecular weight size markers are myosin (200 kD), phosphorylase b (97.4 kD) and bovine serum albumin (68 kD).

Western

8 cell



detectably by the A'IAP and A1.3 antisera (Figs. 5, 6 and7). This protein is recognized faintly, if at all, by the anti-6016 antiserum.

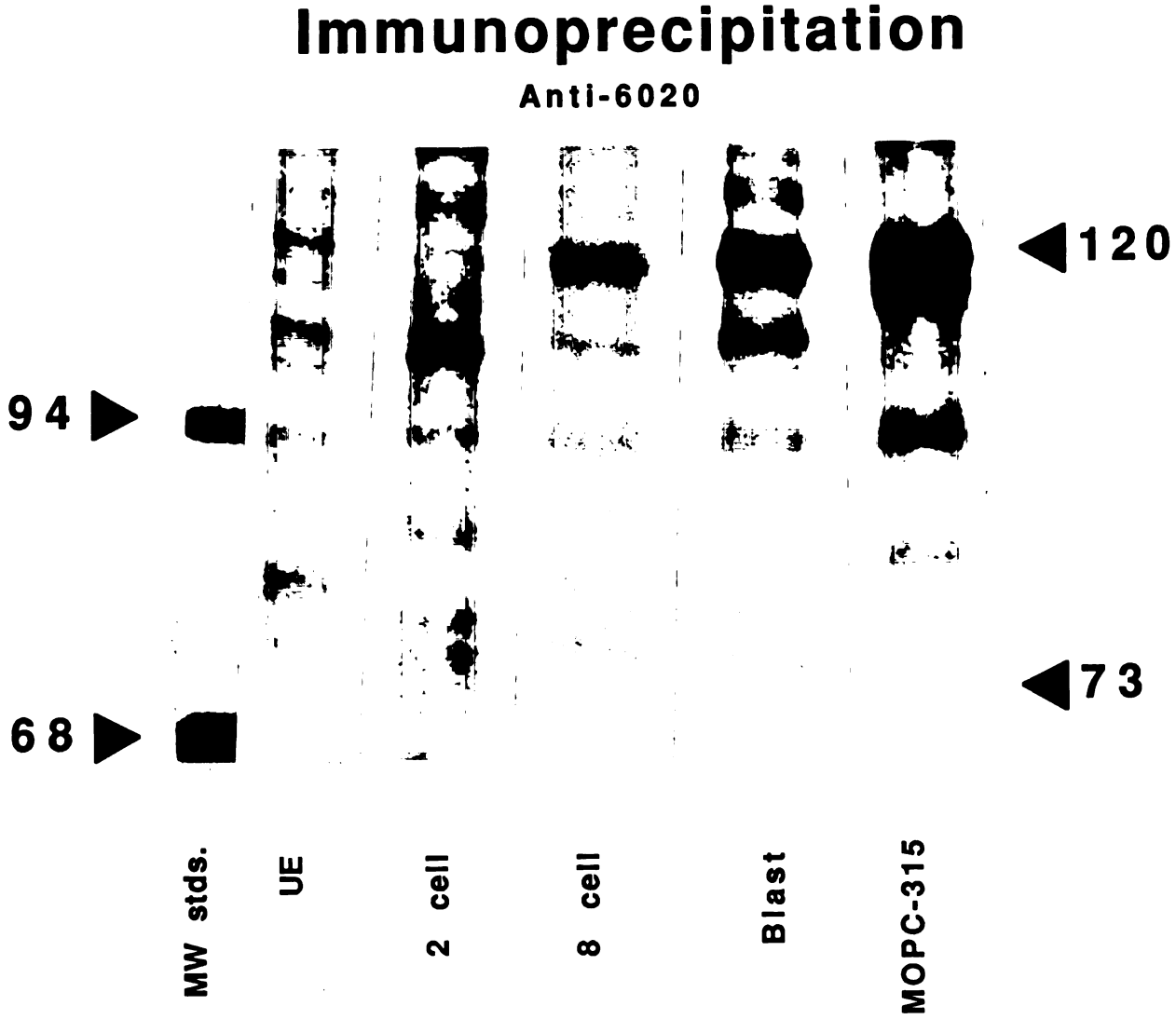
technical note

After transfer, the non-specific sites on the blots are routinely blocked with a solution of "BLOTTO" (Johnson etal.,1984) and 5% normal serum of the same species of the secondary biotin-conjugated antibody. The biotin and/or streptavidin reagents react strongly with a set of proteins on the protein blots of preimplantation embryos, regardless of the specific primary antibody which is used (Fig. 6 and 7). The recognition of these proteins is so consistent that they can be used as orientation markers for other proteins on the blots. The fact that these proteins are recognized even on control strips which have not been incubated with any primary antiserum and on control strips which have seen only the streptavidin and biotin reagents but not on control strips which have seen only substrate solution suggests that they are not merely recognized as non-specific proteins by all rabbit sera nor are they non-specifically stained by interaction with the substrate solution. From these results I suspect that these mouse embryo proteins are reacting with biotin and/or streptavidin and may be seen by others who examine mouse embryo proteins using this type of detection system.

Immunoprecipitation of ^{35}S-methionine Labeled Proteins

The anti-peptide antiserum anti-6020 was used to immunoprecipitate IAP-related proteins from ^{35}S-methionine labeled embryos and an IAP producing cell line, MOPC 315 (Fig. 8).

Fig. 8. Immunoprecipitation of IAP-related antigens from cells and embryos. Anti-6020 antiserum was used to immunoprecipitate IAP-related antigens from ^{35}S-methionine labeled unfertilized eggs, 2-cell embryos, 8-cell embryos, blastocysts and MOPC-315 cells. The 120 kD IAP *gag-pol* fusion protein is prominent in immunopreipitates of the 8-cell and blastocyst stages and of the MOPC-315 plasmacytoma cell line. The 73 kD IAP *gag*-related protein is recognized faintly by this particular antisera in all stages. Molecular weight size markers are phosphorylase b (97.4 kD) and bovine serum albumin (68 kD).


Immunoprecipitation
Anti-6020
94
68
120
73
MW stds.
UE
2 cell
8 cell
Blast
MOPC-315

A protein of 120 kD. is prominent is the immunoprecipitates of the 8-cell and blastocyst embryos whereas this protein is not obvious in the unfertilized egg or 2-cell stage. The IAP core protein, p73, can also be detected in the immunoprecipitates from all stages but the anti-6020 antiserum is not particularly good at precipitating this protein from either neoplastic cell lines (Kuff et al., 1986) or embryos.

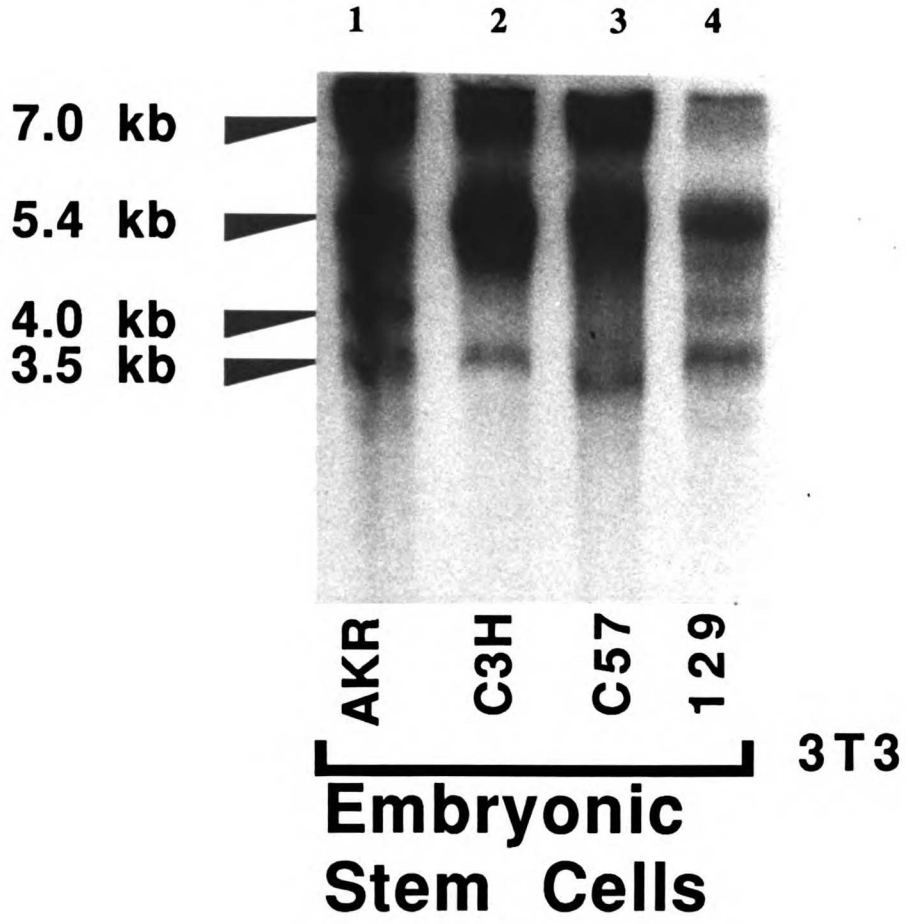
Embryonic Stem Cell RNA Blot

An northern blot of RNA prepared from embryonic stem cell (ESC) lines derived from four different strains of mice (AKR, C3H, C57/BL and 129) was hybridized with the pMIA1 IAP DNA probe (Lueders and Kuff, 1980) which recognizes sequences in common to both type I and type II IAP transcripts (Fig. 9).

The AKR and C57/BL ESC lines express at high levels and approximately equal amounts the 7.2 kb and 5.4 kb IAP transcripts (lanes 1 and 3). The expression in the C57/BL ESC line contrasts with the IAP expression in normal thymus from this strain (Kuff and Fewell, 1985) in which the 7.2 kb transcript is undetectable and the 5.4 kb transcript is present at only low levels. The C3H ESC line also expresses both of these RNAs but the 5.4 kb transcript is present at a higher level than the 7.2 kb transcript (lane 2). The 129 ESC line expresses the 5.4 kb transcript in amounts approximately equal to the other ESC lines but expresses the 7.2 kb transcript only at a very much lower level (lane 4).

Fig. 9. Northern blot of RNA (20 ug each) prepared from embryonic stem cell (ESC) lines derived from blastocysts of four different strains of mice (AKR, C3H, C57/BL and 129). The blot was hybridized to the IAP probe, pMIA1, which recognizes sequences in common to both type I and type II IAP transcripts. Transcripts of 7.0 kb, 5.4 kb, 4.0 kb and 3.5 kb are detected. RNA of the 3T3 cell line is included as a control.

Embryonic Stem Cell Northern



In addition to these larger IAP RNAs, at least two smaller transcripts can be detected even at short exposure times for the autoradiographs (Fig. 9). These transcripts also show strain related variability. RNAs of 4.7 kb and 4.0 kb correspond to the transcripts of type IIA and type IIB IAP genes, respectively. The AKR and 129 ESC lines express both the 4.7 kb and 4.0 kb transcripts (lanes 1 and 4). The C3H ESC line expresses only the 4.0 kb transcript (lane 2) and the C57/BL ESC line expresses neither of these transcripts, but does express a slightly smaller 3.5 kb transcript (lane 3).

RNA Slot Blot

RNA slot blots of 2-cell through blastocyst stage embryos were hybridized with type I and type II specific ^{32}P-labeled DNA probes. The blots were then stripped and rehybridized with an alpha-actin cDNA probe (Minty et al., 1981). On the basis of densitometric tracings of the autoradiographs the relative levels of transcription of type I and type II IAP genes were estimated at the 8 cell and blastocyst stages relative to known transcript levels of alpha-actin from the data of Giebelhaus et al. (1985). The alpha-actin hybridization was used to normalize the signal from the IAP hybridization to deduce the relative amount of RNA present. This was done since slightly different numbers of embryos were used for each sample on the slot blot and the efficiency of RNA extraction from the different stages could conceivably vary.

The results demonstrate that both type I and type II IAP genes are transcribed at all stages examined, although the signal from the

Fig. 10. RNA slot blots of 2-cell (2C) through blastocyst (BL) stage embryos hybridized with type I and type II specific ^{32}P-labeled IAP DNA probes. The slots represent 3000 2-cell embryos, 1060 8-cell (8C) embryos and 609 blastocysts. The type I IAP probe was the 1.0 kb Sst to BamH1 sequence clone and the type II probe was pTAII.1. The autoradiograph shown here was overexposed to show the presence of 2-cell transcripts. Densitometry was performed on an autoradiograph which was exposed to give signals for the stages which were within a linear range of detection.

Embryo RNA Slot Blot

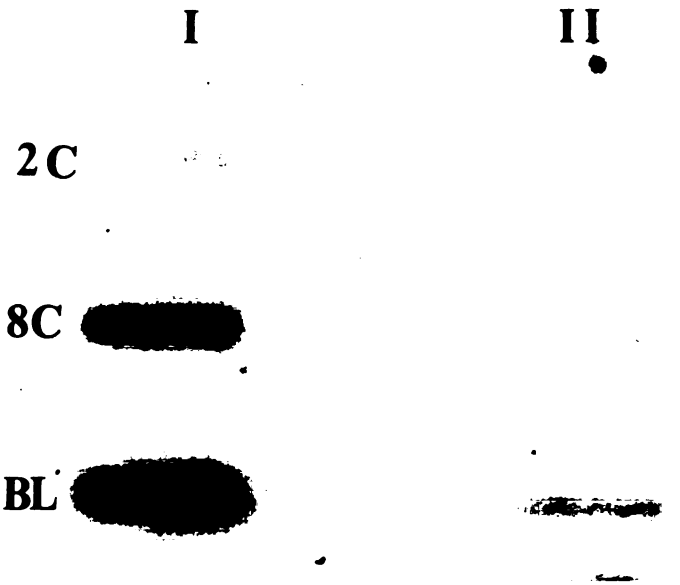


Fig. 11. Relative transcription of type I IAP sequences at the 8-cell and blastocyst stages on a per-embryo and a per-cell basis. Embryo RNA slot blots from Fig. 10 were stripped and rehybridized with a beta-actin probe. The autoradiographs were scanned with a laser-densitometer. The ratio of the amount of beta-actin RNA per embryo at the 8-cell stage to the blastocyst stage is 1 to 13.3. From the ratio of the densitometric signals from the 8-cell and blastocyst samples on the autoradiographs of the slot blots hybridized with the actin probe, the ratio of embryo RNA equivalents between these stages was deduced. This factor was used to normalize the densitometric signals from the same slot blots hybridized to the IAP probes and these values were used to compare relative transcription of type I IAP sequences at these stages.

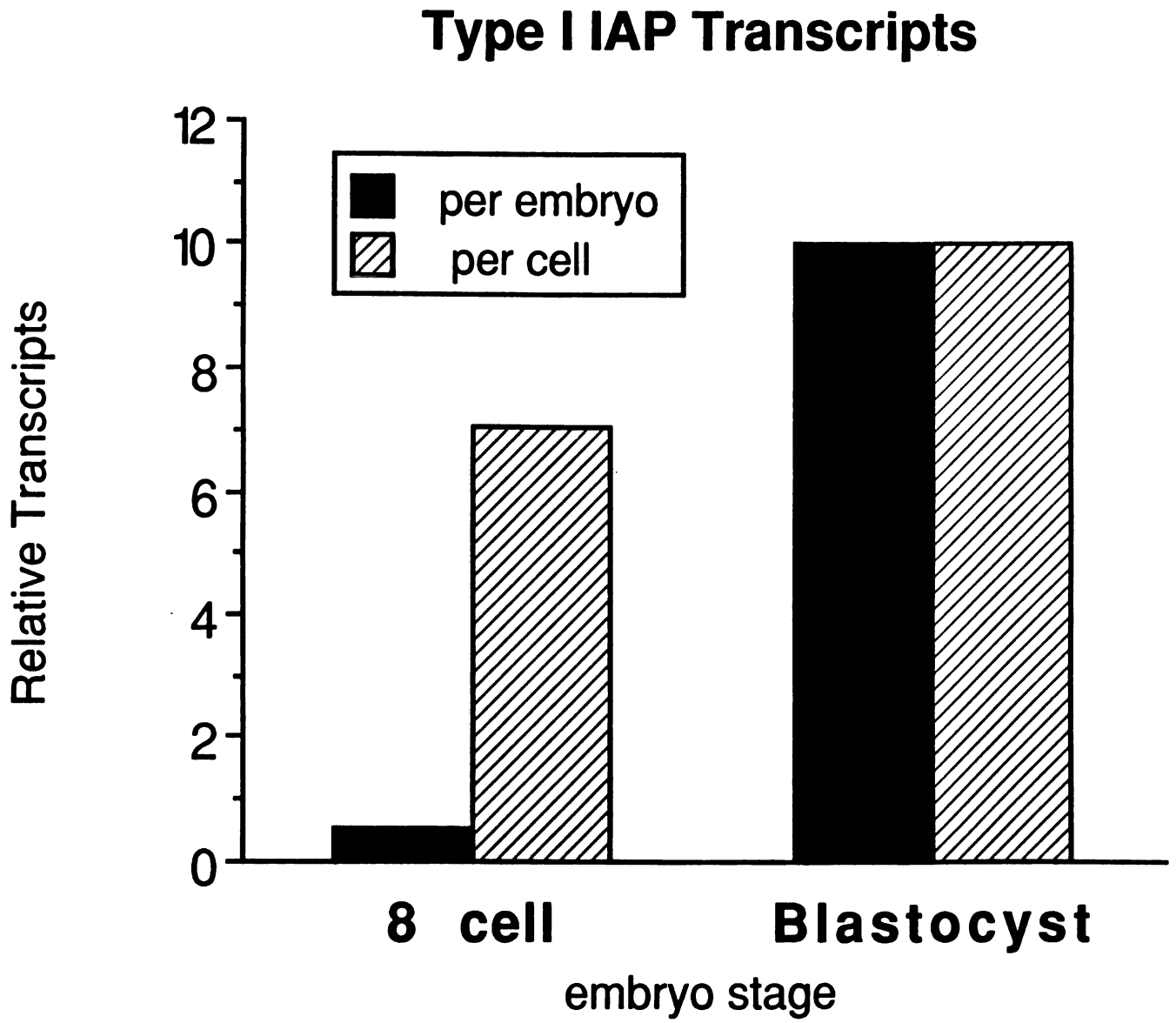
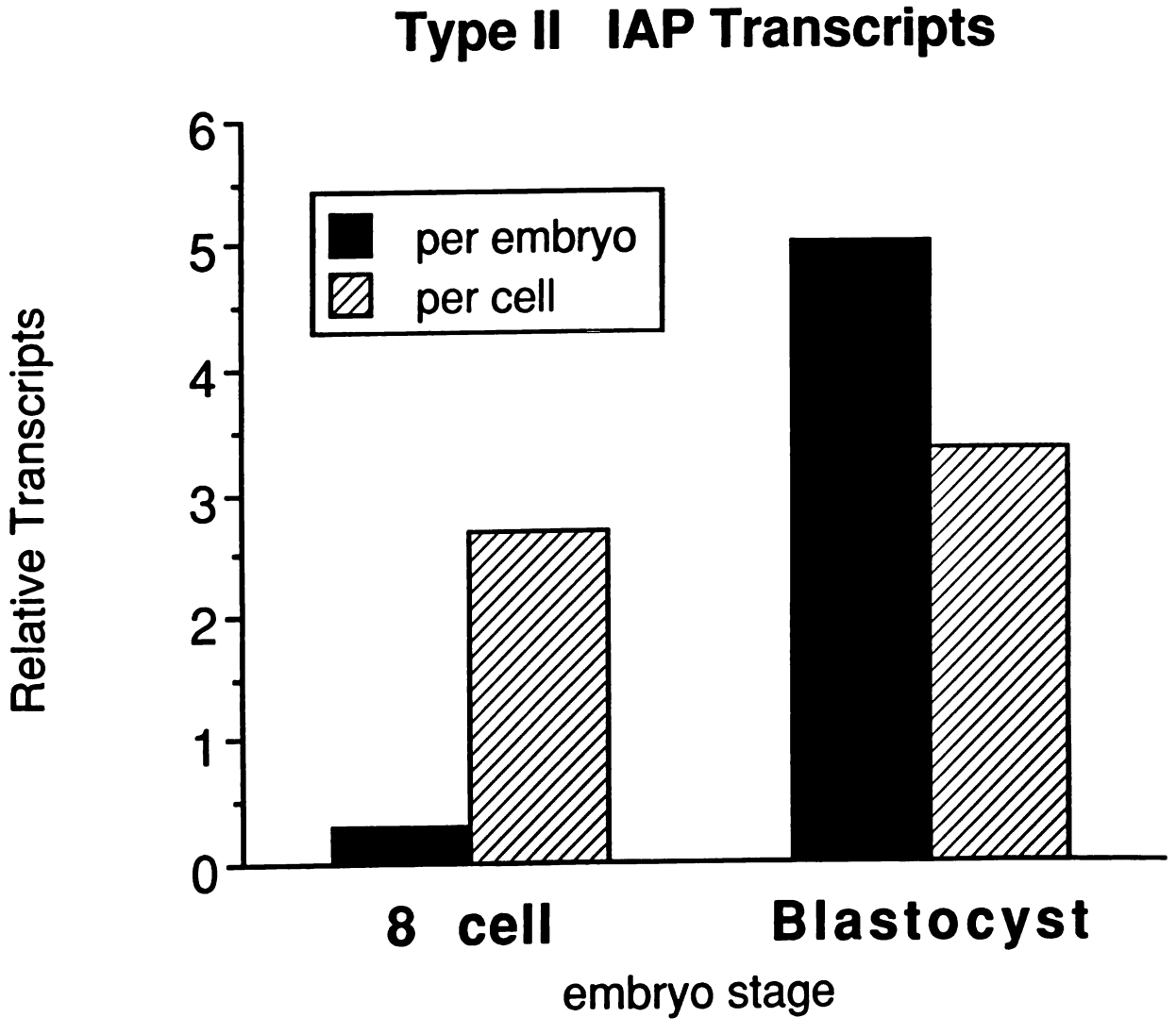

Type I IAP Transcripts
12
10
8
6
4
2
0
per embryo
per cell
Relative Transcripts
8 cell
Blastocyst
embryo stage

Fig. 12. Relative transcription of type II IAP sequences at the 8-cell and blastocyst stages on a per-embryo and a per-cell basis. Embryo RNA slot blots from Fig. 10 were stripped and rehybridized with a beta-actin probe. The autoradiographs were scanned with a laser-densitometer. The ratio of the amount of beta-actin RNA per embryo at the 8-cell stage to the blastocyst stage is 1 to 13.3. From the ratio of the densitometric signals from the 8-cell and blastocyst samples on the autoradiographs of the slot blots hybridized with the actin probe, the ratio of embryo RNA equivalents between these stages was deduced. This factor was used to normalize the densitometric signals from the same slot blots hybridized to the IAP probes and these values were used to compare relative transcription of type II IAP sequences at these stages.


Type II IAP Transcripts
Relative Transcripts
6
5
4
3
2
1
0
per embryo
per cell
8 cell
Blastocyst
embryo stage

2-cell stage was too faint for a detailed analysis (Fig. 11). By comparison with the hybridization to the alpha-actin probe the relative amounts of type I and type II IAP transcripts were determined (Figs. 12 and 13). Although the absolute amount of transcription seems to increase on a per-embryo basis from the 8-cell through the blastocyst stage for both type I (Fig. 12) and type II (Fig. 13) sequences, when cell number for each stage is taken into account it can be seen that the amount of transcription on a per-cell basis is similar (Figs. 12 and 13).

PCR Blot and Gel

The ethidium bromide stained agarose gel (Fig. 14) shows PCR products of the predicted sizes; 417 bp for type I IAP amplified sequences (lanes 2 and 3), 242 bp and 292 bp for type II IAP amplified sequences (lanes 4 and 5). The last lane is a contol amplification using primers which span an exon/intron border of the beta-actin gene (lane 6). These beta-actin primers used in PCR give products which can be distinguished as amplifications of cDNA templates or genomic DNA templates on the basis of size. The PCR product of amplification of the cDNA is 243 bp. and the PCR product of amplification of the genomic DNA is 373 bp, larger by the size of the 130 bp. intron sequence.

The Southern blot (Fig. 15) demonstrates a number of embryo cDNA samples which were examined for genomic DNA contamination in this way. Some of these samples (lanes 1-4 and 6-8) show only a single PCR product of 243 bp. indicating the amplification of the

Fig. 13. PCR products of the amplification of embryo **cDNA** and **RNA** contaminated with genomic DNA. Embryo **cDNA** (lanes 2, 4 and 6) and **RNA** (lanes 3 and 5) from the same preparation were amplified with type I IAP primers, AP1 and AP3, (lanes 2 and 3), type II IAP primers, AP1 and AP2 (lanes 4 and 5) and actin primers (lane 6). The molecular weight size marker is a 123 bp DNA ladder (lane 1). Although genomic DNA contamination is present in these samples, as evidenced by amplification products in the RNA lanes, this contamination is not detectable at the single copy level, as seen by the presence of only the cDNA actin amplification product (243 bp) in lane 6.

Amplification of Genomic DNA-contaminated Samples

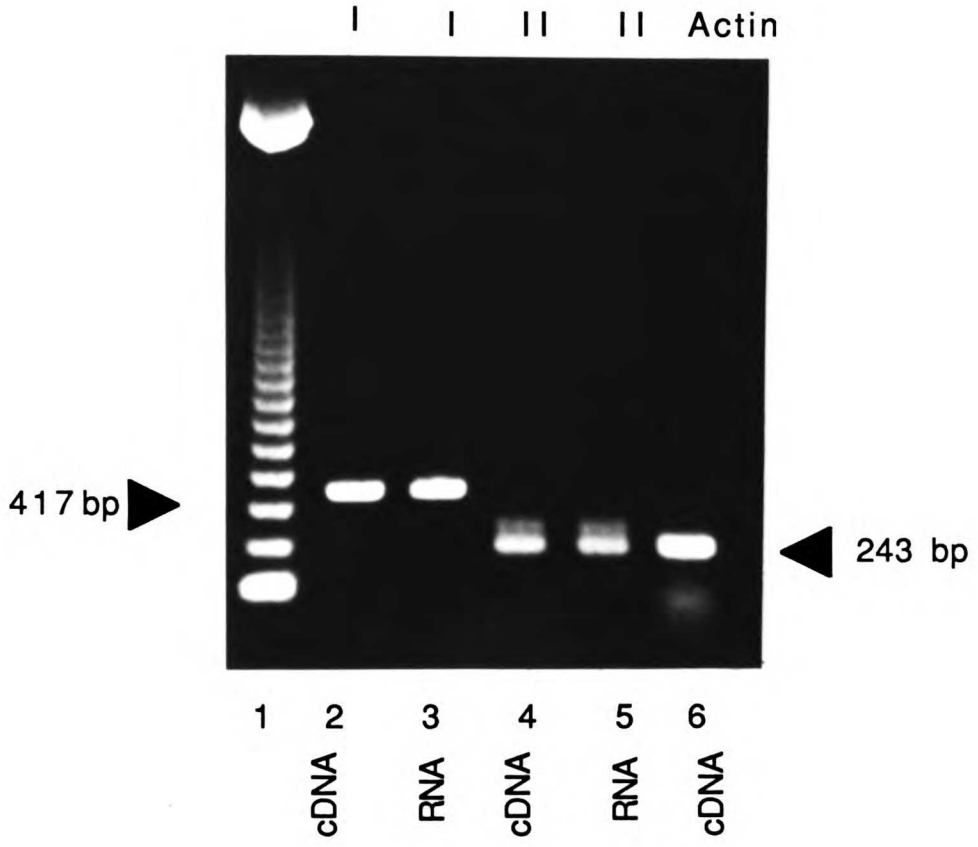
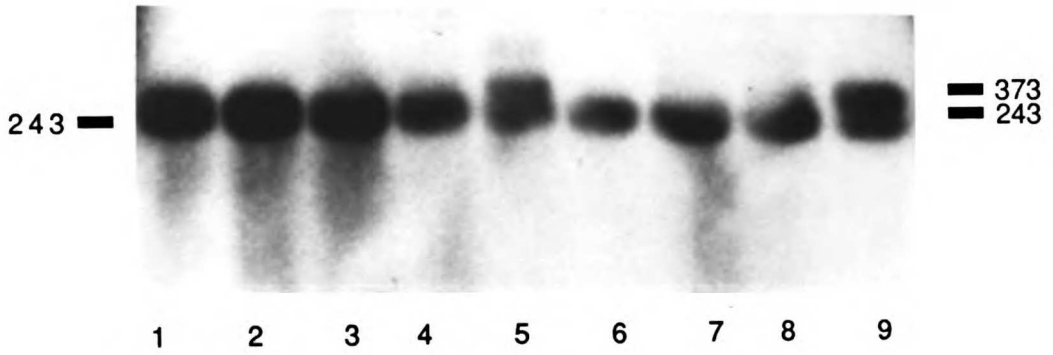


Fig. 14. Southern blot of PCR products of an amplification of various preparations of embryo cDNAs using beta-actin primers which span an exon/intron border. The 243 bp band is the PCR product of the amplification of the beta-actin cDNA (all lanes) and the additional 373 bp band is the PCR product of the amplification of the genomic beta-actin DNA (lanes 5 and 9), larger by the size of the 130 bp intron.

Amplification of Embryo cDNAs with Actin Primers



beta-actin cDNA while other samples (lanes 5 and 9) show two PCR products of 243 bp. and 373 bp. indicating amplification of both the cDNA and the contaminating genomic beta-actin DNA. The problem is that IAP sequences are present in 1000 copies per haploid genome and these genomic DNA sequences could act as template in the PCR. A negative result in examination of the sample for genomic DNA contamination at the level of sensitivity of detection of a single copy gene is inadequate in this case. Lane 6 in the ethidium bromide stained agarose gel (Fig. 14), in fact, represents an "RNA control" sample contaminated with genomic DNA which is not detected at the single copy level by the actin amplification. Therefore, a more appropriate control is to use the hydrolyzed RNA sample and IAP primers to amplify these multicopy gene sequences which are present in any contaminating genomic DNA that may be inadvertantly present in the RNA used to synthesize the cDNA templates.

PCR Stage Gels

Embryo cDNA samples which proved to be free of genomic DNA contamination were examined for type specific sequences by PCR (Fig. 16). Type I sequences are present at all stages, from the unfertilized egg through blastocyst stage (lanes 2-5), while the presence of type II sequences varies according to stage (lanes 12-14). Both type II PCR products, the 242 bp and the 292 bp, are present in the unfertilized egg, the 8-cell embryo and the blastocyst (lanes 12, 14 and 15). At the 2 cell stage the type II sequences are undetectable by ethidium bromide staining (lane 13), however, when

Southern transfers are hybridized with ^{32}P labeled type II DNA probes it can be seen that smaller of the two type II products (242 bp) is present (Fig. 17).

Fig. 15. Detection of IAP mRNA by amplification of mouse unfertilized egg (UE) and embryo cDNAs by the polymerase chain reaction (PCR) using type I and type II specific IAP primers. The PCR products are shown on ethidium bromide stained agarose gels. Type I amplification product (417 bp) from UE (lane 2), 2C (lane 3), 8C (lane 4) and BL (lane 5). Type II amplification products (242 bp, 292 bp) from UE (lane 12), 2C (lane 13), 8C (lane 14) and BL (lane 15). Control amplification from UE (lane 7), 2C (lane 8), 8C (lane 9) and BL (lane 10).

Polymerase Chain Reaction

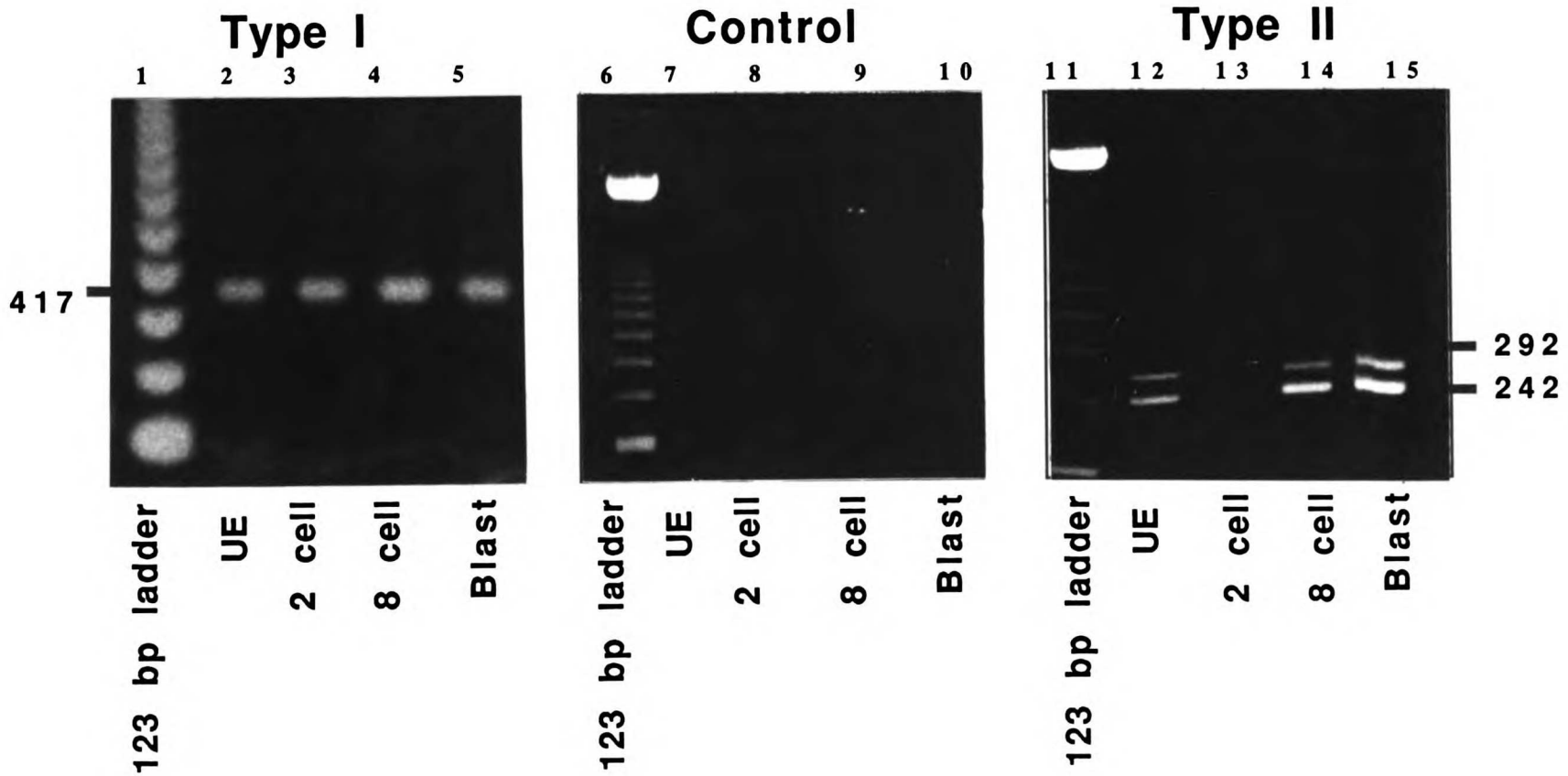
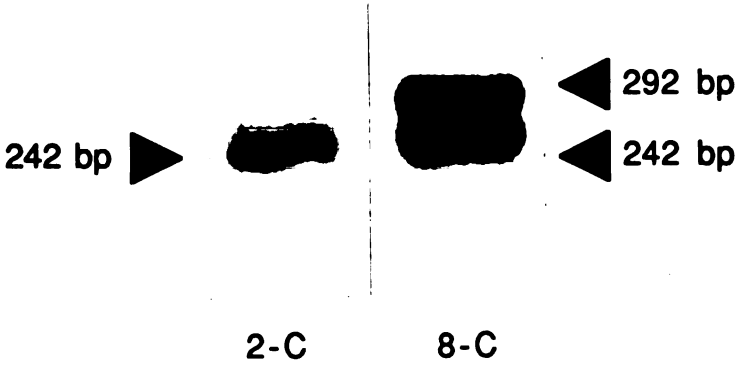


Fig. 16. Southern transfer of PCR products of an amplification of 2-cell (2-C) and 8-cell (8-C) embryo cDNA samples with type II IAP primers hybridized with a type II specific IAP probe, pTAII.1. Two PCR products of 292 bp and 242 bp are detected in the 8-cell amplification while only the 242 bp PCR product is detected in the 2-cell amplification.

Southern Blot of Type II PCR Products



DISCUSSION

The results suggest that throughout the course of preimplantation development, from the unfertilized egg through blastocyst stage, IAP-related proteins are present in the mouse embryo. Both the 73 kD *gag* protein (Figs. 5,6,7 and 8) and the 120 kD *gag-pol* fusion protein (Fig. 8) are detected by the anti-IAP antisera used, although the different antisera vary in their ability to detect these proteins.

Moore and colleagues (1986) have shown that the reaction of the peptide antisera with electrophoretically purified p73 in an enzyme-linked solid-phase immunoassay has endpoint dilutions of 1/40000 for anti-6020 and anti-6021, and 1/2500 for anti-6016. The titers of the anti-peptide antisera in that assay correspond in general to their activity against p73 on Western blots of preimplantation embryos seen here, with the weakest reactivity seen with the anti-6016. (Figs. 5 and 6).

All antisera have in common the recognition of p73, but additional proteins are also recognized by the different antisera (Figs. 5 and 6). The presence of IAP-related proteins on the surface of preimplantation embryos was suggested by Huang and Calarco (1981b) on the basis of indirect immunofluorescence studies using the A'IAP antiserum. However, anti-6020 does not detect surface antigens on whole mount embryos and it now seems likely that the

69 kD and 67 kD proteins recognized by A'IAP in addition to the 73 kD protein, contribute to this surface reaction. Perhaps the 69 kD and 67 kD proteins are not related to IAPs but rather to other proteins from the immunogen, for example proteins of the ER membrane which comprise the outer shell of the particles. These are not removed completely by deoxycholate treatment (Kuff and Lueders, unpublished observation; Huang and Calarco, 1981b). The 77 kD and 75 kD proteins that were recognized by A'IAP antiserum by immunoprecipitation at the UE through blastocyst stages (Huang and Calarco, 1981a) were not recognized by the other anti-IAP antisera, either on blots or in immunoprecipitates. This suggests that these antigens are also reacting with some component of the A'IAP antiserum which is not IAP-related. The lack of detection of p120 by any of the anti-IAP antisera on protein blots of these stages suggests either that this protein is present in very small amounts or that it is altered in its antigenicity during some step in sample preparation for the protein blot technique.

Both the p73 and p120 can be immunoprecipitated from ^{35}S-methionine-labeled preimplantation embryos indicating that these proteins are being actively synthesized during the preimplantation period. Embryos of all stages examined, from the unfertilized egg through blastocyst, synthesize the 73 kD protein while the 120 kD protein is synthesized only from the 8 cell through blastocyst stage (Fig. 8). The ability of anti-6020 to immunoprecitate p120 in embryos of these stages is greater than that of the other anti-peptide antisera, and greater than that of A'IAP.

It may not be surprising that the A'IAP is poor at recognizing IAP-related proteins as compared to the anti-peptide antisera. As one of the final steps in its preparation the antiserum was absorbed three times with crude membrane homogenates of adult mouse livers, kidneys, and spleens. Subsequently, it has been reported that these tissues express IAP sequences at low levels (Kuff and Fewell, 1985). For example, in BALB/c liver IAP poly(A) RNA represents 0.05% of the total poly(A) RNA. Corresponding values for kidney and spleen are 0.1% of the total poly(A) RNA for each tissue. By comparison, IAP poly(A) RNA in thymus represents 0.5% of the total poly(A) RNA and in IAP producing neoplastic cell lines IAP poly(A) RNA represents 8% of total poly(A) RNA. Therefore it is possible that many of the antibodies directed against IAP-related protein were removed, unwittingly, from the antiserum during the absorption step.

The detection of p120 was particularly interesting since it has been reported by Piko, Hammons and Taylor (1984) that an IAP transcript of 5.4 kilobases can be detected in RNA of blastocysts. The 120 kD protein has been shown to be a *gag-pol* fusion protein translated from the deleted type IΔ1 IAP gene transcript of 5.4 kb It seems possible, therefore, that the transcription of IΔ1 IAP elements during preimplantation development in the mouse is responsible for the presence of the 120 kD protein detected by the various anti-IAP antisera. The demonstration by PCR that type I IAP

transcripts are present throughout preimplantation development is consistent with this hypothesis.

By immunoprecipitation anti-6020 recognizes the p120 but does not strongly recognize the p73 of N4 or MOPC 104E cells (Kuff etal., 1986) or of embryos as seen here. The anti-6020 antiserum does, however, recognize p73 in an enzyme-linked solid-phase immunoassay (Moore etal., 1986). Therefore the access of the anti-6020 antiserum to p73 may be different in these two different types of assays due to some conformational change in the protein.

In summary, I have not detected any IAP-related proteins present specifically and only at the 2-cell to 8-cell stages when formed IAPs are abundant. On the contrary, an additional IAP-related protein, p120, is synthesized by the embryo at a time when particles are becoming scarce or absent.

Embryonic Stem Cell Blots

The IAP transcripts display strain related variability in the sizes of transcripts and the relative proportions of the transcripts in ESC lines (Figs. 9 and 10). RNAs of size 7.2 kb and 5.4 kb correspond to the transcripts of type I and type IΔ1 IAP genes, respectively. The strain-related variability of IAP transcripts reported in the thymus (Kuff and Fewell, 1985) and strain-related variability seen here in embryonic stem cell lines argues for caution in extending conclusions about patterns of IAP expression from cells or embryos of one mouse strain to another.

The data from the embryo RNA slot blot (Fig. 11) represents the first evidence for the transcription of type II IAP sequences in preimplantation mouse development. My analysis of relative transcription (Figs. 12 and 13) corroborates and extends the data of Piko, Hammons and Taylor (1984) in which the amount of ^{3}H-labeled IAP-related RNA was assayed by hybridization to filter-bound cloned IAP genes. They found that the rate of synthesis of IAP RNA per embryo increases about 30 fold during preimplantation development but that the rate of synthesis per cell remains about the same, about 4 femptograms per cell over a 5 hour period.

Polymerase Chain Reaction (PCR)

The results indicate that IAP transcripts vary during preimplantation development in the mouse (Fig. 16). Sequence data predicts a PCR product of 417 bp for transcripts derived from type I IAP genes. This product is present at all stages examined, from the unfertilized egg through blastocyst. Sequence data predicts PCR products of 242 bp and 292 bp for transcripts derived from type II IAP genes. Both these products are present at all stages other than the 2-cell stage. At the two-cell stage, when formed particles are abundant, only the smaller product of 242 bp is present (Fig. 17).

It must be added that since non-polyadenylated RNAs are not copied into cDNA by the use of oligo dT primers the IAP transcripts detected by this method represent only messenger RNAs which are polyadenylated. It has been reported that although the majority of

IAP RNA in blastocysts is polyadenylated, a substantial fraction (~ 40%) lacks poly (A) sequences (Piko etal., 1984). This RNA could be examined by the use of cDNA which was made with random hexamer oligonucleotide primers which would not bias the copying of RNA to only polyadenylated species.

A survey of the literature reveals that there is no factor or modification which correlates absolutely with IAP expression. It is clearly not possible to make conclusions about the degree of IAP sequence expression solely on the basis of the morphologically observable particles and it remains a formal possibility that particles could form without an RNA genome. Particle formation seems to be regulated at levels in addition to that of transcription. Several studies illustrate this point. Treatment with 5'-azacytidine, which inhibits methylation, induces a general hypomethylation of the genome and has been shown to increase the number of IAP-positive cells and the number of particles per cell section in two embryonal carcinoma and differentiated cell lines (Hojman-Montes de Oca et al., 1984). On the other hand, Morgan and Huang (1984) found that Msp/HpaII sites throughout the IAP sequence are hypomethylated in IAP-negative 3T3 cells so hypomethylation is a necessary but not sufficient state for IAP expression. Kuff and Lueders (1988) have pointed out that although no consistent relationship between overall methylation state of genomic IAP sequences and intracisternal A particle expression in various cell lines is observed this should not be surprising in view of the large number of IAP elements in the

genome and the possibility that IAP formation may result from transcription of only a small minority of competent genes.

There is no information on the degree to which the genome is methylated during the mouse preimplantation stages which would be of most relevance to the observed IAP expression. It has been shown that mouse sperm DNA is highly methylated with respect to structural genes although satellite DNA sequences are less methylated in sperm than in adult tissues (Waalwijk and Flavell, 1978; Sanford et al., 1985), and that mouse oocyte DNA is undermethylated with respect to repetitive DNA sequences (minor satellite and MIF) (Sanford et al., 1985). These repetitive sequences were also shown to be undermethylated in extraembryonic cell lineages compared with primitive ectoderm derivatives or adult tissues (Chapman et al., 1984). The DNA of the trophoblast of rabbit embryos has also been shown to be comparatively undermethylated (Manes and Menzel, 1981).

The preimplantation embryo has the capacity to methylate DNA sequences as demonstated in experiments by Stuhlman et al. (1981). M-MuLV retroviral sequences introduced into preimplantation embryos were methylated whereas these sequences remained unmethylated when introduced into day 8 post-implantation embryos. It is possible therefore that the embryo uses methylation and demethylation as controls of gene expression early in development and it would be interesting to know the methylation status of transcriptionally active IAP sequences during this time.

A case in which the enhanced expression of IAP sequences does not result in a concomitant increase in particle production is the effect of interferon on an MSV-transformed fibroblast cell line (Emanoil-Ravier et al., 1985). The authors found that in contrast with IAP RNA and protein elevation, a significant reduction in the number of intracisternal A particles was observed.

A study by Morgan and Huang (1987) on F9 teratocarcinoma cells also illustrates the point of multiple levels of regulation. In the undifferentiated F9 embryonal carcinoma cells IAP expression, as judged by particle number and RNA levels, is absent, while in the parietal endoderm-like cells, differentiated from the F9-derived embryoid bodies as a result of retinoic acid treatment, both particles and IAP transcripts are numerous. However, when isolated nuclei from these two different cell types are examined, the levels of IAP transcription are similar. The authors suggest that the observed increase in IAP expression in parietal endoderm cells results from a stabilization of IAP transcripts encapsulated in newly formed particles.

Although all mice thus far examined have been shown to produce IAPs during early embryogenesis there is considerable interstrain variation with respect to the level of their production (Yotsuyanagi and Szollosi, 1981). Szollosi and Yotsuyanagi have categorized different strains of mice as either high or low producers on the basis of the numbers of identifiable particles seen in

morphometric studies on electron micrographic sections. They have also shown that in genetic crosses between high- and low-producing strains that low production character is dominant in all the hybrids, regardless of whether the low-production character is of paternal or maternal origin (Szollosi and Yotsuyanagi, 1985). They interpret these results to indicate a paternally introduced regulatory mechanism that is active as early as the two-cell stage. Levels of IAP transcripts were not examined in these studies.

These results are interesting to reconsider in light of the data presented here. In PCR experiments examining the types of IAP transcripts present at various stages of preimplantation development it was found that type I and type II transcripts are expressed at all times from the unfertilized egg through the blastocyst stage. The only difference in the pattern of the transcripts occurs at the two-cell stage when the smaller of the two type II transcripts (242 bp) is absent. Since the two-cell stage corresponds to the peak of particle production, one possible hypothesis is that the presence of both type II transcripts at all other stages somehow prevents particle production and that when one of the type II transcripts is absent particles form from the existing transcripts.

The type II transcripts, which are deleted with respect to the type I transcripts, may be defective for particle formation in and of themselves but they may be capable of occupying the cellular machinery which is responsible for particle formation. This machinery could be the ribosomes, signal recognition particles,

binding sites on endoplasmic reticulum membrane or some other accessory component. By EM data (Calarco and Brown, 1969) it is clear that the ER is rather primitive in its morphology during early stages of development and there is evidence that mouse eggs and early embryos have limited translational capacity. The bulk of ribosomes in the ovulated mouse egg are inactive in protein synthesis (Bachvarova and De Leon, 1977) and the spare translational capacity of fertilized mouse eggs for injected mRNAs is extremely limited (Ebert and Brinster, 1983). From the 2-cell stage onwards there is a rapid synthesis of both ribosomal proteins (LaMarca and Wassarman, 1979) and ribosomal RNA (Piko and Clegg, 1982).

Type II sequences could be defective in any number of the steps along the way to particle formation. For instance, a defective signal sequence may prevent association of the hypothetical nascent type II protein with the endoplasmic reticulum, or if it succeeds in associating with the endoplasmic reticulum membrane it may fail to be cleaved properly due to a defect in the peptide cleavage site (Meitz and Lueders, 1987). Another possibility is that a type II encoded protein, although correctly processed through the endoplasmic reticulum, is unable to be packaged into a particle due to lack of a proper packaging signal or some defect in secondary structure which causes improper folding

For these or other reasons, type II transcripts and/or p120 may occupy and block the particle forming apparatus of the embryo

without actually forming any IAPs themselves, thereby limiting the number of IAPs which can be formed by the type I transcripts and the p73 present. From this point of view the hybrid condition of low particle production, seen by Szollosi and Yotsuyanagi (1985) could be seen as a result of increased transcription of the type II sequences with respect to the parental high producer. Accordingly, the effect of interferon on the transformed cell line studied by Emanoil-Ravier et al. (1985) could be to increase expression of type II sequences and thereby diminish particle formation by competent type I sequences. It should be possible to test this hypothesis with the use of PCR. Alternatively, the particles seen at the 2-cell stage may represent a different virus which shares antigenic determinants with the IAP. It is also possible that maturation of the ER in the embryo somehow blocks virus production.

FUTURE DIRECTIONS

IAP-related genetic information makes up to about 0.3% of the total cellular DNA in *Mus musculus*. What can the expression of these repetitive genes tell us about development? Ultimately, it has brought to our attention that these vertebrate retrotransposons are expressed during early developmental stages, at a time which may help explain how they have come to be dispersed throughout the murine genome and may hint at their involvement in maintainance of genomic fluidity in murine evolution. Although the information encoded by IAP genes may be only tolerated by, the embryo their expression during the earliest stages of development may be useful in designing strategies to examine other genes active during this same period which are developmentally important.

At least four possible reasons for IAP gene expression during early development can be envisioned. First, the location of particular IAP genes with respect to actively transcribed cellular genes may bring the IAP sequences under the control of cellular regulatory sequences. Secondly, particular IAP genes could be located in regions that were favored for transcription due to some local conformation or opening of the chromatin (possibly due to hypomethylation). Thirdly, the regulatory sequences of the IAP genes, themselves, may be responding to the presence of transacting factors present during this time. The presumption in this case is that the embryonic cell produces these factors to ensure the expression of cellular genes. Fourthly, the IAP transcripts or related proteins may

actually have a function, although preliminary evidence suggests that is an unlikely possibility.

A low level of IAP transcription is constitutive for many normal mouse tissues but enhanced IAP expression is characteristic of three situations: 1) certain normally proliferating cells, such as immature thymocytes, 2) neoplastic mouse cell lines, and 3) early embryonic development. It has been suggested that this general association between cell proliferation and IAP expression might be mediated by the enhancing action of viral or cellular oncogenes on the promoter activity of IAP LTRs (Luria and Horowitz, 1986). Nuclear oncogene products (c-myc, SV-40 T antigen, p53 and E1a) have been shown to have an activating effect on the LTR of IAP genes in experiments in which an IAP LTR - CAT (chloramphenicol acetyltransferase) construct is introduced into cell lines which express one of these oncogene products (Luria and Horowitz, 1986). In light of these findings, IAP expression from endogenous genes may be a useful indication of cellular oncogene activity in situations of normal proliferation such as early embryonic development and implantation (Luria and Horowitz, 1986).

An important caveat which complicates the interpretation of IAP expression as an indication of oncogene activity involves the multicopy nature of IAP genes. The approximately 1000 copies of IAP genomic elements are distributed among most, and perhaps all chromosomes (Lueders and Kuff, 1977.). These elements vary in size and coding potential and are variably methylated. Ascertaining

whether the expression of a particular element is due to position, modification or the activating influence of a trans-acting factor would therefore be difficult, if not impossible. The use of a construct which linked an IAP LTR to an indicator gene, such as beta-galactosidase, could circumvent these complications.

On the basis of methylation studies, a region within the IAP LTR consisting of three clustered Hha sites 5' of the RNA start site has been postulated to contain a methylation-sensitive binding site for trans-acting factors (Feenstra et.al., 1986). An LTR-beta galactosidase construct could be engineered to examine the function of this region in embryos and transgenic mice.

Viruses have often provided useful probes for cellular regulatory events. Evidence for the presence of cellular homologues of the viral products which could mediate these regulatory events is accumulating. Examples include: 1) demonstration of N-myc expression in post-implantation embryos (Jacobovits et.al., 1985), of c-myc in proliferating cytotrophoblast cells of the placenta, but not in the non-proliferating syncytiotrophoblast cells (Pfeifer-Ohlsson et.al. 1984), in F9 EC stem cells, but not in differentiated F9 endodermal cells (Griep and DeLuca, 1986) and 2) demonstration of an "E1a-like activity" in F9 EC stem cells, but not in differentiated F9 endodermal cells (La Thangue and Rigby, 1987), and an "E1a-like activity" present in morulae and blastocysts prior to hatching and implantation (Suemori et.al., 1988).

The expression of these factors in preimplantation embryos has not been fully characterized. For those factors whose gene sequence is known, such as c-myc, the technique of PCR would be able to characterize expression in preimplantation embryos. For those viral factors for which homologous cellular proteins are suspected, the sequence of conserved regions with known function, such as E1a region 3, could be used to design primers which may be able to fish out these cellular genes by PCR under conditions of lowered stringency. Alternatively, a strategy utilizing the IAP LTR could be envisioned. cDNAs from embryo stages would be put into expression vectors and the expressed proteins would be screened for their ability to bind to a labeled IAP LTR nucleic acid probe. Recently several groups have begun to identify proteins that bind to LTR sequences of IAP genes (Falzon and Kuff, 1988a,b; Zierler and Huang, 1988). We may hope that within the next several years these types of studies will allow us to understand more about the expression of the endogenous retroviral genes during normal preimplantation development.

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