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Two alkaline phosphatase genes are expressed during early development in the mouse embryo

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Summary

Alkaline phosphatase (AP) activity is stage specific in mouse embryos and may be associated with compaction and separation of trophectoderm from inner cell mass in preimplantation development. We previously sequenced a cDNA and two mouse AP genes that could contribute to the AP activity in embryos. Oligonucleotide primers were constructed from the three sequences and used in the reverse transcription-polymerase chain reaction technique to establish that two of the three AP isozymes are transcribed during preimplantation development. The predominant transcript (E-AP) is from a gene highly homologous to the human tissue-specific APs, but different from the mouse intestinal AP. Tissue non-specific (TN) AP also is transcribed, but there is approximately 10 times less TN-AP than E-AP transcript. The TN-AP isozyme is the predominant transcript of 7 to 14 day embryos and primordial germ cells. A switch in predominance from E-AP to TN-AP must occur during early postimplantation development. This study establishes a framework for experiments to determine the functions of the two isozymes during preimplantation development.

Key words: alkaline phosphatase, mouse embryo, preimplantation, polymerase chain reaction, primordial germ cells.

Introduction

The first morphogenetic event of mouse embryogenesis is an epithelialization of blastomeres that begins at the 8-cell stage (Ducibella, 1977; Pratt *et al.* 1982). Polarization of cell-surface and cytoplasmic components is followed closely by compaction, the maximization of cell surface contacts and the formation of intercellular junctions. Epithelialization accompanies differentiation of mouse cells into the trophectoderm and inner cell mass (ICM) of the blastocyst. There is no indication that these events are controlled by maternal mRNAs. Transition from maternal to embryonic control in mouse embryos, when maternal mRNA is degraded and transcription from the zygotic genome begins, occurs at the 2-cell stage and precedes the events of compaction (for review, see Schultz, 1986). Cell polarity and position of a cell are important to its fate (Rossant, 1977; Johnson and Ziomek, 1981; Balakier and Pedersen, 1982; Ziomek and Johnson, 1982). Therefore interactions between cells or between cells and the environment, and thus the cell surface molecules modulating these interactions, are important.

Alkaline phosphatases (AP, orthophosphomonoester phosphohydrolase, EC 3.1.3.1) constitute a small family of cell surface enzymes that may play a role in morphogenetic or differentiative processes. In general, the physiological functions of the alkaline phosphatases are unknown. Their importance has been inferred by their ubiquitous species expression, their stage and tissue specificities in vertebrate development and their association with active centers of differentiation or morphogenesis. By histochemical staining, AP activity appears at the late 2- or 4-cell stage of mouse embryos and appears to be restricted to the ICM of late blastocysts and primary ectoderm of pregastrulation embryos (Mulnard and Huygens, 1978; Izquierdo *et al.*

1980; Ziomek, unpublished results). There are foci of high AP activity in postgastrulation embryos, including primordial germ cells (Chiquoine, 1954; Mintz and Russell, 1957).

Most of what is known about the AP isozymes comes from studies with human adult tissues (for reviews, see Fishman, 1974; Sussman, 1984; Millán, 1988; Harris, 1989). In man, the cDNAs and genomic sequences for four isozymes have been determined, and they code for the enzymes referred to as tissue non-specific or liver/bone/kidney AP (TN-AP) and the tissue-specific APs intestinal AP (I-AP), term placental AP (Pl-AP) and testicular germ-cell AP (GC-AP). There is immunological cross-reactivity between human I-AP, Pl-AP and GC-AP, but no cross-reactivity between any of these and the TN-AP form. The AP proteins are coded for by mRNAs that are approximately 2.4 kb long. I-AP, Pl-AP and GC-AP are highly homologous (a minimum of 90 % sequence identity at the amino acid level), whereas TN-AP is only 57 % identical to I-AP at the amino acid level. In mice, only two isozymes have been previously distinguished by biochemical techniques, one characteristic of the intestine and the other of liver, bone, kidney and placenta (Goldstein *et al.* 1980). A separate placental form of the enzyme, equivalent to human Pl-AP, does not appear to exist in mice (Goldstein *et al.* 1980; Terao and Mintz, 1987).

Kim *et al.* (1989) suggested that multiple forms of AP are expressed during mouse preimplantation development, but they could not distinguish between secondary processing differences and multiple genes. Lepire and Ziomek (1989) identified an AP in preimplantation mouse embryos with an activity profile similar to that of human Pl-AP and mouse I-AP, although not identical to either. They also reported that the preimplantation form of AP cross-reacted immunologically with the human Pl-AP and GC-AP isozymes. Although Lepire and Ziomek did not detect a TN-AP isozyme in preimplantation embryos, Merchant-Larios *et al.* (1985) only found enzyme activity of the TN-AP form in postimplantation embryonic tissues, including primordial germ cells from day 7 to day 15. Mouse embryonal carcinoma (EC) cells express an AP with an electrophoretic, inhibition and immunological pattern like that of mouse TN-AP (Hass *et al.* 1979).

The findings reported above do not conclusively establish which AP genes are transcribed during development. The first step toward assigning a role to these cell surface molecules during preimplantation development is a definitive characterization of the gene expression pattern of this small multi-gene family. We previously sequenced a cDNA of an AP from mouse Nulli SCC1 (nullipotent) EC cells (Hahnel and Schultz, 1989). This AP is in the TN-AP isozyme group by comparison to the human sequences and is the same as that expressed in mouse term placenta (Terao and Mintz, 1987). We also have completed the sequences of two mouse genomic AP clones with close homology to human I-AP, Pl-AP and GC-AP (Manes *et al.* 1990). Using the three sequences, we constructed oligonucleotides and used the reverse transcription-polymerase chain reaction (RT-PCR) technique to determine which of the three isozymes is transcribed during early mouse development and in other selected mouse cells including primordial germ cells. We found that preimplantation embryos do not express I-AP and do express TN-AP. However, the predominant AP mRNA of preimplantation embryos (E-AP) is transcribed from a third mouse AP gene similar to the germ cell AP of human testis, although E-AP is not transcribed by mouse primordial germ cells.

Materials and methods

Embryo recovery

Random-bred CD1 mice (Charles River Breeding Laboratories) were used throughout the study, except for the gel analysis of AP activity. For these gels, CF1 females (Harlan Sprague Dawley) mated to B6SJL/J males (Jackson Laboratory) were used due to their superior embryo yield per female (Chatot *et al.* 1990). Procedures for superovulation and recovery of oocytes and preimplantation embryos were as described in Giebelhaus *et al.* (1983). Procedures for recovery of postimplantation embryos (day 7.5 to 9.5) from naturally mated females were as described in Hogan *et al.* (1986).

Isolation of primordial germ cells

Gonads with mesonephros were dissected from 12 to 14 day CD1 fetuses using fine watchmaker's forceps in a large volume of calcium- and magnesium-free phosphate-buffered saline (CMF PBS). The gonads were separated from the mesonephros using glass needles and pooled (usually from a litter size of 10–12) regardless of sex. The tissues were washed briefly with CMF PBS, followed by two transfers to 100 μl drops of trypsin/pancreatin (0.5 %/2.5 %) with incubation at 37 °C for 30 min in the second drop. After incubation, an equal amount of flushing medium-1 (Spindle, 1980) containing 10 % fetal calf serum was added and the suspension was aspirated in and out of a Gilson pipetteman with a yellow tip. The suspension was put in a 15 ml centrifuge tube with 5 ml of L15 medium containing serum and centrifuged at room temperature for 5 min at 1000 revs min^{-1}, the supernatant poured off and the pellet resuspended in 5 ml of 0.025 % bovine serum albumin in CMF PBS and centrifuged again as above. The cell pellet was resuspended in 200 μl of 0.025 % BSA and loaded on top of a 12 ml 0.5–10 % BSA gradient in an inverted 12 ml sterile plastic syringe fitted with silicon tubing for fraction collection. The cells were allowed to separate for 3 h at room temperature, and 1 ml fractions were collected from the bottom. Fractions 1–6 were pooled and kept separate from the pool of fractions 7–12. The two pools were centrifuged, and the pellets stored at −20 °C in 100 μl 4 M of guanidinium thiocyanate containing 20 μg *E. coli* RNA as a carrier.

RNA, cDNA and DNA preparation

RNA was extracted from pools of 100 to 300 preimplantation embryos in the presence of 5–10 μg of *E. coli* ribosomal RNA (Boehringer-Mannheim), as described by Giebelhaus *et al.* (1983). Embryos in 50 μl of buffer (0.2 M NaCl, 1 mM EDTA, 25 mM Tris, pH 7.4) were vortexed with an equal volume of phenol, and phases were separated by centrifugation. The aqueous phase was re-extracted with chloroform, recovered after centrifugation and precipitated in ethanol. RNA pellets were dissolved in 3 μl of sterile distilled water and the entire sample was reverse transcribed (see below).

To extract RNA from 7.5 to 9.5 day embryos, pools of 15 embryos at each stage were homogenized in 100 to 200 μl of cold buffer composed of 0.2 M NaCl, 0.05 M Tris–HCl, pH 7.2, 0.005 M $MgCl_2$ and 0.5 % Triton X-100. The homogenate was centrifuged to pellet nuclei and cell debris and the resultant supernatant adjusted to 10 mM EDTA and 1 % sodium dodecyl sulfate (SDS) by addition of appropriate amounts of concentrated stock solutions. The supernatant was extracted two times with an organic phase of phenol:chloroform (1:1) and the resultant aqueous phase was extracted a final time with chloroform:isoamyl alcohol (24:1). Following centrifugation to separate phases, RNA was precipitated from the aqueous phase by addition of 2 volumes of cold absolute ethanol and storage at −20°C overnight. The RNA pellet was collected by centrifugation, washed with 80 % ethanol and dissolved in water for use in the RT-PCR procedure.

Genital ridges, EC cells and adult tissues were dissolved in 4 M guanidinium thiocyanate, 0.1 M Tris (pH 7.5), 1 % 2-mercaptoethanol and 0.5 % sodium lauryl sarcosinate (MacDonald *et al.* 1987). RNA was separated from protein and DNA by centrifugation through cesium trifluoroacetate (1.51 g ml^{-1}) according to the manufacturer's protocol (Pharmacia).

RNA was reverse transcribed at 43°C for 60 min in a final volume of 20 μl containing 500 μM each dNTP, 100 μg ml^{-1} oligo (dT), 30 i.u. AMV reverse transcriptase (Molecular Genetics), 1 i.u. μl^{-1} human placental ribonuclease inhibitor (RNAguard, Pharmacia), 50 mM Tris (pH 8.3), 10 mM $MgCl_2$ and 10 mM dithiothreitol (Hagen *et al.* 1988). The reaction volume was increased to 100 μl with 10 mM Tris (pH 7.5), 1 mM EDTA and extracted once each with an equal volume of phenol and chloroform and precipitated in ethanol. The pellet was dissolved in 30 μl of 2 mM Tris (pH 8.2).

Mouse genomic DNA was extracted from tail tissue according to the method of Hogan *et al.* (1986).

Primer pairs

The mouse TN-AP primer pair was constructed from the sequence of a cDNA in a Nulli SCC1 EC cell library (Hahnel and Schultz, 1989). The 5′ primer is identical to nucleotides 1283 through 1302 (20-mer), and the 3′ primer is the reverse complement of nucleotides 1591 through 1612 (22-mer). The E-AP and I-AP primers were derived from the corresponding genomic sequences (Manes *et al.* 1990). The 5′ E-AP primer is identical to nucleotides 2464 through 2484 (21-mer) of the E-AP gene and anneals within exon IX; its 3′ partner is the reverse complement of nucleotides 3082 through 3101 (20-mer) and primes within exon XI. The 5′ I-AP primer is the same as nucleotides 1624 through 1644 (21-mer) of the I-AP gene and anneals within exon VI, and the 3′ primer is the reverse complement of nucleotides 2281 through 2302 (22-mer) and primes within exon IX. These oligonucleotides were prepared by the Regional DNA Synthesis Laboratory, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada. The 5′ c-*kit* primer is the same as nucleotides 4774 through 4796 of the published sequence (Qiu *et al.* 1988), and the 3′ primer is the reverse complement of nucleotides 4954 through 4976. The c-*kit* primers were prepared by the Biomolecular Resource Center, University of California, San Francisco.

PCR

PCR was performed as described by Saiki *et al.* (1988) and Rappolee *et al.* (1988, 1989). One-tenth (3 μl or material equivalent to approximately 10–30 embryos) of a reverse transcription reaction (or 600 ng genomic DNA) was amplified in a final volume of 50 μl (100 μl for genomic DNA) containing 10 mM Tris (pH 8.4), 50 mM KCl, 5 μg nuclease-free BSA, 1 mM $MgCl_2$ (2 mM for genomic DNA), 1 μM each sequence-specific primer, 1 μM each dNTP and 1 U per 50 μl *Thermus aquaticus* (Taq) DNA polymerase (Amplitaq, U.S. Biochemical). The mixture was overlaid with mineral oil, presoaked at 94°C for 3 min and subjected to 25 to 60 cycles of amplification in a DNA thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of denaturation at 94°C, cooling to 55°C over 2 min (to anneal the primers) and extension at 72°C.

Products of the PCR reaction (one-fifth reaction volume) were resolved electrophoretically on 2 % agarose gels along with a small molecular weight DNA ladder (pBR322 cut with *Hin*fI), stained with ethidium bromide and photographed with Polaroid 52 film.

To quantify PCR product, amplification was performed as above except that 0.8 μCi α-^{32}P-dCTP (3000 Ci $mmol^{-1}$, ICN) was added to each reaction in the first trial and 2 μCi per reaction in the second trial. One-fifth of the reaction product was separated and stained in 2 % agarose as above, the bands were excised, and radioactivity was determined in liquid scintillant (Econofluor-2, NEN). Background levels were determined from agarose slices removed from the area above the bands.

For restriction enzyme analysis, amplified PCR products were ethanol precipitated and resuspended in the appropriate restriction enzyme buffer. Digested and undigested PCR products were separated on 6 % nondenaturing acrylamide gels and stained with ethidium bromide or electrophoretically transferred to nylon membranes (Nytran, Schleicher and Schuell). A Southern blot of putative TN-AP PCR product from blastocysts was hybridized to an RNA synthesized from the 2.4 kb TN-AP cDNA (Hahnel and Schultz, 1989) with T7 RNA polymerase (Pharmacia) in the presence of α-^{35}S-UTP (1350 Ci $mmol^{-1}$, NEN). Conditions for hybridization and posthybridization washes were as described previously (Hahnel *et al.* 1986) except that the SDS concentration was 0.75 % and dextran sulfate was omitted.

Gel analysis of AP activity

Various stage embryos were collected in 10 μl of Hanks' Balanced Salt solution containing 0.66 mg ml^{-1} polyvinylpyrrolidone (Sigma) and frozen at −70°C. All samples were mixed with an equal volume of 2× sample buffer (0.125 M Tris, 4.6 % SDS, 10 % glycerol and 5 % β-mercaptoethanol). Non-boiled samples of 250 embryos per lane, human placental AP (hPLAP) and bovine kidney AP (TN-AP) (Sigma) were loaded onto duplicate 7.5 % SDS–polyacrylamide mini slab gels (0.4 % SDS). After electrophoresis, one gel served as the control, while the other was incubated for 1 h at room temperature in 1 mM levamisole (Sigma). Both gels were stained overnight for AP activity with the Promega Protoblot AP detection system (Promega Blotec) in the absence (control) and presence of 1 mM levamisole (inhibited). The AP stain buffer contained 0.1 M Tris, 0.1 M sodium chloride, and 0.005 M $MgCl_2$ at pH 9.5. The staining solution contained 0.33 mg nitro blue tetrazolium (NBT) and 0.165 mg 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) per ml of AP stain buffer.

Results

Design of oligonucleotide primer pairs

We designed oligonucleotide primer pairs that bracket target sequences specific for each of the three mRNAs (cDNAs) that encode mouse APs (Fig. 1). There are 6

TISSUE NON-SPECIFIC AP TARGET SEQUENCE

```
                                                GTGGATAGACCCCCCGGGGC  20
                                                5' PRIMER
                  I(700bp)
AACTCCATCTTTGGTCTGGCTCCCATGGTGAGCGACACGGACAAGAAGCCCTTCA  75

CGGCCATCCTATATGGTAACGGGCCTGGCTACAAGGTGGTGGACGGTGAACGGGA 130

                  I(500bp)
AAATGTCTCCATGGTAGATTACGCTCACAACAACTACCAGGCCCAGTCCGCTGTT 185

CCCCTGCGCCATGAGACCCACGGTGGAGAAGACGTGGCGGTCTTTGCCAAGGGCC 240
  Hha I                                          Apa I

CGATGGCACACCTGCTTCACGGCGTCCATGAGCAGAACTACATTCCCCATGTGAT 295

GGCGTATGCCTCCTGCATTGGGGCCAACCTTGACC                      330
                  3' PRIMER
```

EMBRYONIC AP TARGET SEQUENCE

```
                                          CGCACCAGTGAGCAGGACACG  21
                                          5' PRIMER
ATGATCCTTGTCACTGCCGACCACTCTCACGTCTTCTCTTTTGGTGGTTACACAC  76

                  I(113bp)
AGAGAGGGGCTTCCATCTTTGGACTGGCTCCCTTCAAGGCTGAGGATGGCAAATC 131

CTTTACCTCGATACTATACGGCAACGGTCCTGGTTACAAGCTCCATAATGGCGCC 186
          Ava II          Alu I     Aha II

                  I(87bp)
CGGGCTGATGTCACTGAGGAAGAGAGCAGCAACCCCACGTACCAGCAGCAGGCCT 261
                                                  Stu I

GTGTACCCCTGTCGTCAGAGACCCACAGCGGGGAGGACGTGGCAATATTCGCGCG 296
                                                Ssp I

TGGCCCACAGGCGCACCTGGTGCACGGAGTTCAGGAGCAGAACTACATCGCGCAC 351

GTCATGGCCTTCGCAGCCTGCCTGGAGCCCTACACTGACTGCGGCCTGGCAT    406

CCCCTGCTGGCCAGAGCAGTGCAGTGAGCCCGGGC                      438
                  3' PRIMER
```

INTESTINAL AP TARGET SEQUENCE

```
                                          GCTGGAACCCCAGACCCGAG  21
                                          5' PRIMER
TATCCAAATGATGCTAATGAGACTGGAACCAGATTGGATGGCAGGAATCTGGTGC  76

                  I(81bp)
AGGAATGGCTGTCAAAGCACCAGGGATCCCAGTATGTTTGGAATCGTGAACAACT 131
          Bam H1

                                          I(129bp)
CATTCAGAAGGCCCAGGATCCGTCAGTGACATACCTCATGGGCCTCTTTGAGCCT 186
      Bam H1

GTAGACACAAAATTTGATATTCAACGAGATCCCCTGATGGACCCATCTCTGAAGG 241
  Acc 1

ATATGACAGAGACGGCCGTGAAAGTGCTAAGCAGGAACCCCAAAGGCTTTTATCT 296

                  I(73bp)
CTTTGTGGAGGGGGGCCGAATCGACCGTGGTCACCATCTGGGCACAGCTTATCTG 351

GCGCTGACTGAGGCTGTGATGTTCGACTTAGCCATCGAGAGGGCC           396
                  3' PRIMER
```

Fig. 1. PCR target sequences from three mouse AP cDNAs. Primer sequences are underlined; arrows indicate intron (I) sites in the genomic sequences, and the intron size is in brackets; arrowheads indicate location of diagnostic restriction endonuclease cleavage sites. The mouse TN-AP genomic sequence was assumed to have the same introns as the human gene (Weiss *et al.* 1988).

to 15 mismatches between primer sequences and their homologues in the other cDNAs. The primers for one cDNA did not bind to cDNA from the other two isozymes under our annealing conditions (55 °C). If the AP mRNA sequenced from EC cells (tissue non-specific, TN-AP) was present, then the appropriate primer pair was expected to amplify a 330 bp piece of DNA that would cut with the restriction endonucleases *Hha*I and *Apa*I. The primer pair constructed for the first genomic sequence (called embryonic, E-AP, see below) was designed to amplify a section of cDNA 438 bp long that would cut with *Aha*II, *Alu*I, *Ava*I and *Ssp*I. Primers constructed for the second genomic sequence (intestinal, I-AP) would amplify a 394 bp piece of cDNA that would cut with *Bam*HI and *Acc*I. The RNA extraction technique that we used excludes DNA, but the primer pairs also were selected to cross intron–exon borders of the genomic sequences (arrows in Fig. 1). Genomic DNA would produce a larger product than the cDNA, allowing us to distinguish between signal due to mRNA and signal due to contaminating genomic DNA. We amplified mouse genomic DNA using the three primer pairs (Fig. 2)). All samples produced DNA of the expected size for the genomic sequence. The smaller size band predicted by mRNA (cDNA) sequence was not amplified in genomic

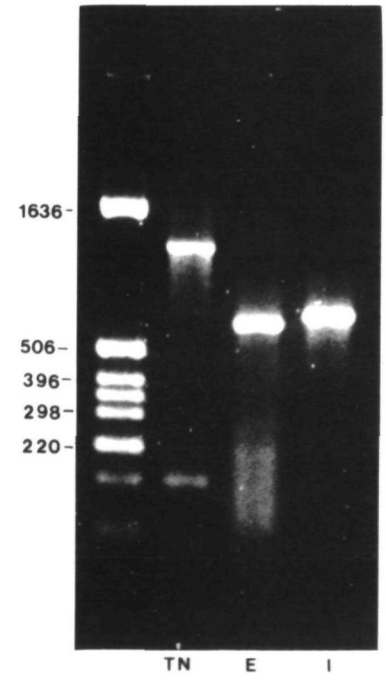

Fig. 2. Mouse genomic DNA amplified with three mouse AP primer pairs. 600 ng of mouse genomic DNA was amplified with primer pairs specific for tissue non-specific AP (TN), embryonic AP (E) and intestinal AP (I), and the DNA fragments resolved on a 2 % agarose gel. Marker DNA (pBR322 cleaved with *Hin*fI) is in the far left lane. The expected fragment sizes (exons plus introns) were: TN-AP, approximately 1530 bp (by analogy to the human TN-AP genomic sequence); E-AP, 638 bp; and I-AP, 679 bp.

DNA samples. Likewise, the larger genomic target sequences were not amplified in any of the RNA preparations, indicating that they were, indeed, free of contaminating DNA sequences (see Figs 3, 5 and 6). A minor band of approximately 150 bp was detected after amplification of DNA with the TN-AP primer pair. We do not know the identity of this minor band, but it is not of the correct size to be derived from either genomic DNA or messenger RNA TN-AP sequences.

Expression of AP mRNAs and protein in preimplantation embryos and selected tissues

The results of PCR amplification of cDNA from preimplantation mouse embryos and selected adult tissues are shown in Fig. 3. E-AP mRNA is present in 2-cell and 8-cell embryos and blastocysts, but not in unfertilized eggs. Our PCR reactions used reverse-transcribed RNA from roughly equivalent numbers of embryos because total embryo mass and protein content do not change greatly during the preimplantation period (Schultz, 1986), and because the exact number of cells in what is morphologically a blastocyst varies. Moreover, within the blastocyst there is more than one cell type that may transcribe AP mRNA. Since we could have missed detecting mRNA in unfertilized eggs because too few cells were extracted to be within RT-PCR detection limits, we also amplified cDNA from eight times the number of eggs (116) as 8-cell embryos (14). E-AP mRNA still was not detected in eggs in this experiment (data not shown).

TN-AP mRNA regularly was detected in 2-cell embryos through blastocysts, although it took more cycles to detect than the E-AP form in all but the blastocyst stage. A TN-AP product was occasionally amplified in unfertilized egg preparations and was the only AP mRNA detected in extracts of whole 7.5, 8.5 and 9.5 day embryos by RT-PCR (data not shown). I-AP mRNA was not present in preimplantation embryos or nullipotent EC cells, but was the major isozyme found in mouse intestinal RNA (thus I-AP). The E-AP fragment was amplified in adult mouse testis and intestine, but not in liver or nullipotent EC cells. TN-AP RNA was detected by PCR in EC cells, liver, testis and intestine.

In each case, the size of the amplified DNA was as

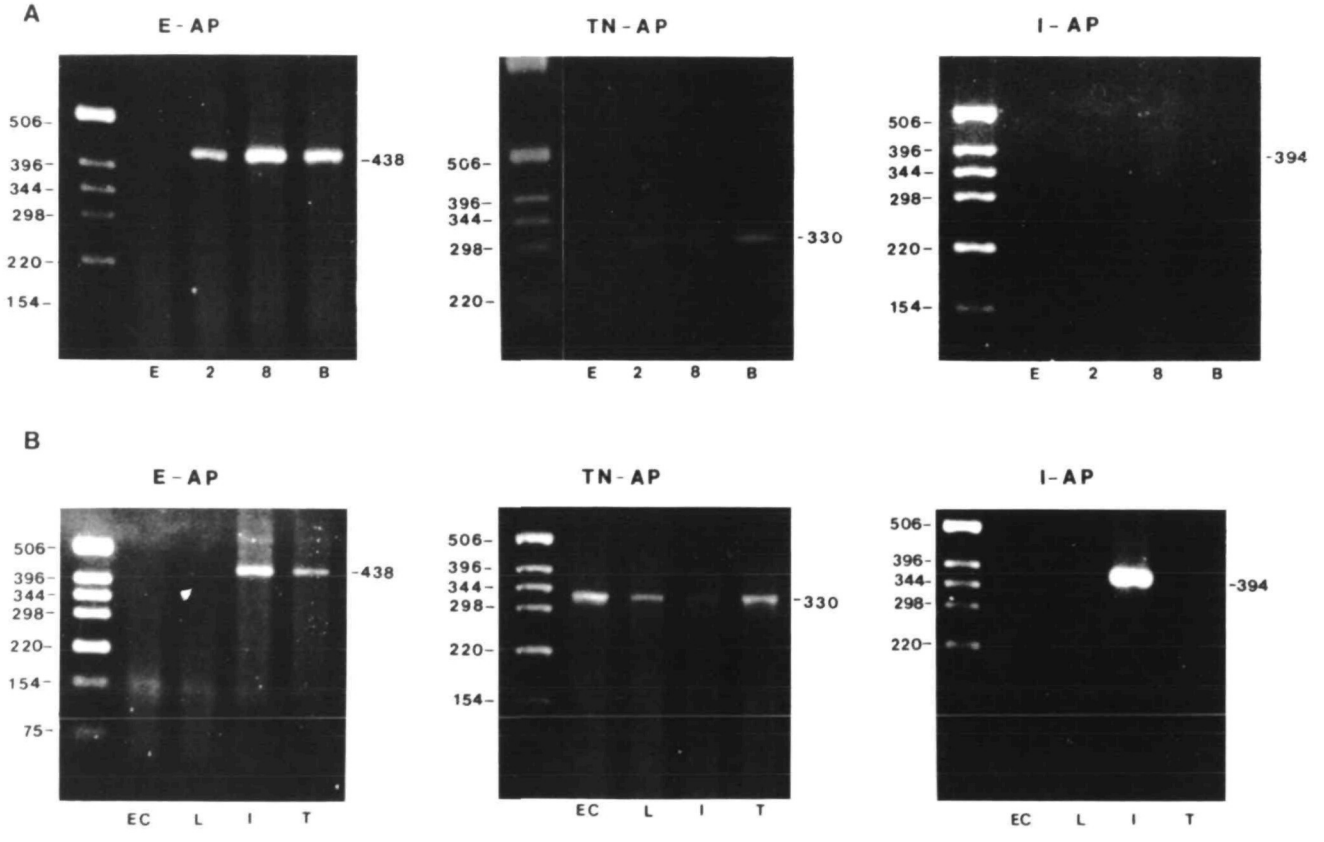

Fig. 3. Expression of AP isozymes in embryonic and adult tissues. (A) RT-PCR results from mouse preimplantation embryos. RNA from unfertilized eggs (E), 2-cell embryos (2), 8-cell embryos (8) and blastocysts (B) was reverse transcribed and amplified by PCR with primers specific for three mouse AP cDNAs. Products were resolved on 2% agarose gels along with molecular weight markers. Size of the PCR product expected from the cDNA sequence of an AP isozyme is indicated on the right of each gel. cDNA from 21 unfertilized eggs, 31 2-cell embryos, 17 8-cell embryos and 14 blastocysts was amplified by RT-PCR with the E-AP and I-AP primers. cDNA from 11 unfertilized eggs, 8 2-cell embryos, 11 8-cell embryos and 7 blastocysts was amplified by RT-PCR with the TN-AP primers. (B) RT-PCR results from mouse cell and tissue types. RNA samples from Nulli SCC1 embryonal carcinoma cells (EC), liver (L), intestine (I) and testis (T) were treated as described above.

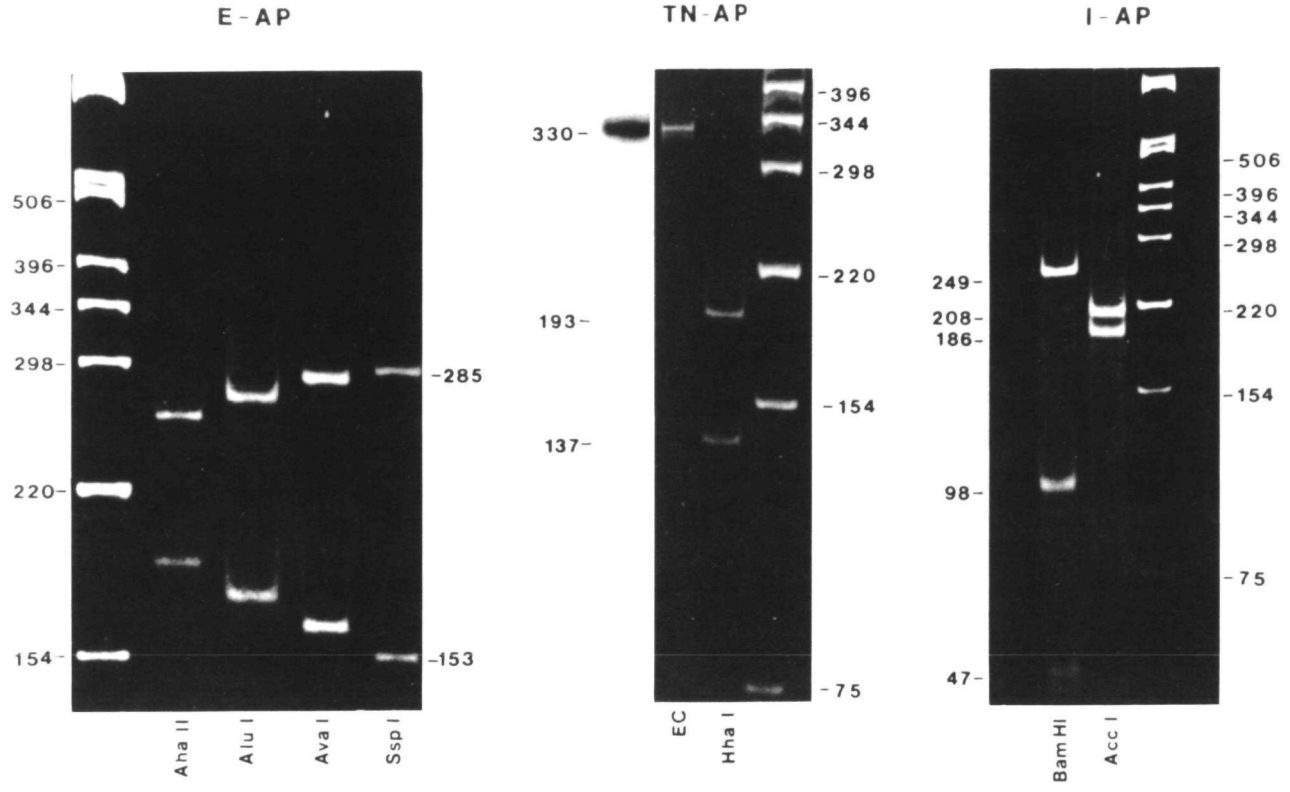

Fig. 4. Identification of AP PCR amplification products by restriction enzyme mapping. cDNA from 8-cell embryos was amplified with the E-AP primer pair, cDNA from blastocysts with the TN-AP primer pair and cDNA from intestine with the I-AP primer pair. The amplified product was cleaved with the indicated restriction enzymes, and the fragments separated on 6% non-denaturing polyacrylamide gels along with molecular weight markers. The PCR product from amplification of an EC cell cDNA with TN-AP primers also was processed as above (EC), transferred to a nylon membrane and hybridized at high stringency to the cDNA clone of mouse TN-AP (left lane of center panel).

expected from the isozyme nucleotide sequence. Further proof of identity was established by restriction enzyme digestion (Fig. 4). The PCR fragment amplified with the TN-AP primers from blastocysts cut with *Hha*I (and *Apa*I, data not shown) as predicted. The fragment also hybridized at high stringency to the original AP cDNA clone from the EC cell library. The E-AP PCR fragment from 8-cell embryos cut with four restriction endonucleases giving the expected patterns, and the I-AP from small intestine cut appropriately with *Bam*HI and *Acc*I.

From the intensity of ethidium bromide staining on gels, we consistently observed more E-AP product than TN-AP product in preimplantation embryos. This visual observation was confirmed by comparing radioactively labeled PCR product from 2-cell, 8-cell and blastocyst-stage embryos. DNA bands were excised from agarose gels and radioactivity was determined by liquid scintillation spectrometry (Table 1). TN-AP PCR product from 2-cell embryo cDNA was detectable only after 40 cycles, whereas E-AP product was detectable after 30 cycles. TN-AP product was 12% of the total AP product at 40 cycles (850 and 6300 cts min^{-1} above background for TN-AP and E-AP, respectively). At the

Table 1. *Incorporation of ^{32}P-dCTP (cts $min^{-1} \times 10^{-2}$) into AP cDNA by RT-PCR*

PCR cycles	25		30		35		40	
Primers	TN-AP	E-AP	TN-AP	E-AP	TN-AP	E-AP	TN-AP	E-AP
cDNA source:								
2-cell embryo			N†	7.6	N	18.0	8.5 (12%)	62.9
8-cell embryo			6.4 (21%)	24.0	10.0 (20%)	39.2	14.8 (16%)	75.8
Blastocyst			6.7 (17%)	33.3	10.7 (13%)	70.9	21.7 (16%)	110.8
Testis	8.1 (55%)	6.7	12.4 (48%)	13.4	23.2 (55%)	19.0	57.3 (64%)	32.1

* Numbers in parentheses represent the percent of total AP PCR fragment incorporation represented by the TN-AP sequence.
† N, not detectable.

8-cell stage, TN-AP product contributed on average 18% to the total AP product; 15% of blastocyst product was amplified from TN-AP mRNA (cDNA). 7% and 13% of 8-cell stage and blastocyst AP product was amplified from the TN-AP isozyme sequence when a different set of RNA preparations was used (data not shown). Both pairs of primers were tested on the same embryo preparations and under the same conditions in each experiment, but mRNAs can be differentially extracted and reverse transcribed, and the primer pairs may have different binding efficiencies. Even though the percentages are approximations of the relative abundance of the two types of mRNA in preimplantation embryos, the results above suggest that 80% to 90% of AP mRNA synthesized by preimplantation mouse embryos is the E-AP isozyme.

To verify that, in addition to expression of both TN-AP and E-AP genes as mRNA, the encoded proteins were also expressed during preimplantation development, 1-cell, 2-cell, 8-cell and blastocyst stage embryos (250/lane) were run on SDS–polyacrylamide gels and stained for AP activity in the presence and absence of 1 mM levamisole. The inhibitor levamisole is specific for TN-AP (Borgers, 1973) and does not inhibit either E-AP (Lepire and Ziomek, 1989) or human Pl-AP. As seen in Fig. 5A, no significant AP activity was detected in 1-cell embryos (a trace of activity is sometimes seen). AP activity increased from the 2-cell to blastocyst stages with the predominant form of AP (a doublet) running ahead of human Pl-AP and showing resistance to inhibition by levamisole (Fig. 5B). The doublet probably represents different levels of glycosylation of the same base molecule as has been seen for many AP isozymes and is not resolved in the 8-cell and blastocyst lanes due to the overloading necessary to visualize the third band of AP. This third minor form of AP, which migrated more slowly than the doublet, was faint at the 8-cell stage and increased in the blastocyst. It was sensitive to levamisole, as was the predominant slower-migrating band of bovine kidney AP and represents the expression of TN-AP in the preimplantation mouse embryo. It therefore appears that both the E-AP and TN-AP mRNAs are translated into protein during preimplantation development and that the relative abundance of each isozyme reflects the deduced relative abundance of their respective mRNAs.

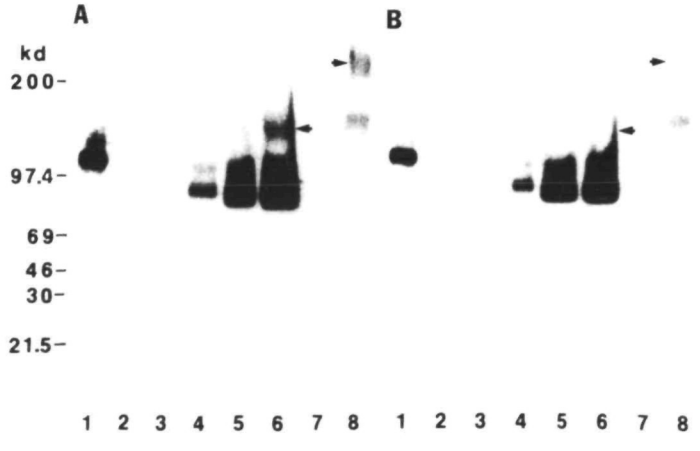

Fig. 5. Gel analysis for AP activity. SDS–polyacrylamide gels stained for AP activity in the absence (A) and presence (B) of the TN-AP inhibitor, levamisole (1 mM). Lane 1, human Pl-AP; lane 3, 1-cell embryos; lane 4, 2-cell embryos; lane 5, 8-cell embryos; lane 6, blastocysts; and lane 8, bovine kidney AP. Lanes 2 and 7 are empty. Positions of the molecular weight markers are indicated. Arrowheads indicate the AP bands that disappear upon levamisole treatment.

Expression of AP mRNA in primordial germ cells

Mouse primordial germ cells (PGC) are one of the few cell types in day 10–14 embryos that histochemically stain for AP activity (Chiquoine, 1954; Mintz and Russell, 1957), and the only cell type to stain for AP activity in genital ridges of day 12–14 embryos. Although Merchant-Larios *et al.* (1985) showed that the AP activity of PGC was of the TN-AP type using specific inhibitors, we found that preimplantation embryos and testicular cells contained E-AP mRNA. Therefore, we used RT-PCR to determine which of the three AP mRNAs are transcribed by PGC. RNA from genital ridges of day 12 and day 13 embryos and gonads from day 14 embryos were screened by the RT-PCR procedure with the three AP primer pairs (Fig. 6A; I-AP not shown). We determined that mouse PGC transcribe TN-AP but not E-AP or I-AP. Cells from day 12 to 14 gonads also were separated on gradients into two cell populations. The two fractions were analyzed by RT-PCR with the AP primer pairs and with a primer pair for the oncogene c-*kit*, a gene presumed to be expressed by germ cells (Mintz and Russell, 1957; Geissler *et al.* 1988; Nocka *et al.* 1989). Fig. 6B shows that TN-AP is expressed in the cell fraction with c-*kit* activity. No PCR amplification products were observed in this fraction with E-AP or I-AP primer pairs.

Discussion

At least three AP genes are expressed in mice, two of which are expressed by preimplantation mouse embryos (see Table 2). The major AP mRNA of preimplantation embryos (E-AP) has close homology to mouse I-AP, but is encoded by a distinct gene (Manes *et al.* 1990). E-AP is not transcribed or translated in unfertilized eggs but is detected in 2-cell stage embryos when the zygotic genome becomes active and is continuously transcribed and translated through the blastocyst stage. Our results indicate that E-AP is not the major AP mRNA of post-gastrulation embryos, nor is it the isozyme expressed by migrating primordial germ cells, although we cannot exclude the possibility that a minor cell population transcribes E-AP in 7.5 to 9.5 day embryos. The temporal pattern of transcription and translation of E-AP correlates with the time that AP activity can be detected by histochemical staining in preimplantation embryos (Mulnard and Huygens, 1978; Izquierdo *et al.* 1980). We do not know yet whether all cells of preimplantation embryos transcribe E-AP, but

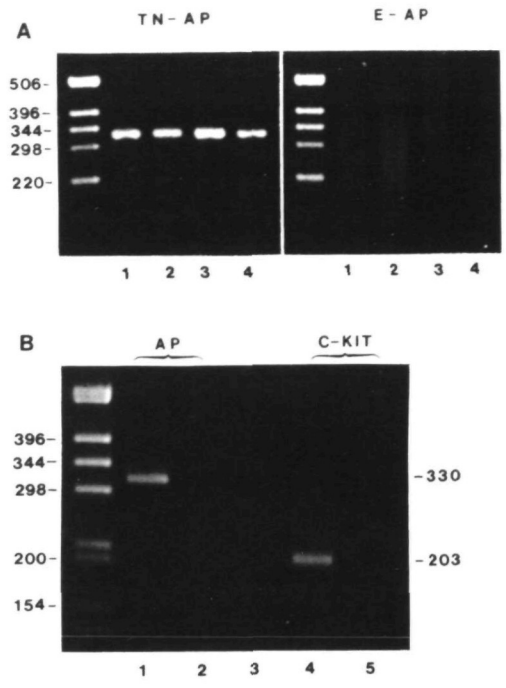

Fig. 6. AP expression in mouse PGC. (A) RT-PCR products from mouse genital ridge RNA were derived as described in the legend to Fig. 3. Lane 1, 12-day genital ridges; lane 2, 13 day genital ridges; lane 3, 14 day gonads; lane 4, 14 day kidney. (B) RT-PCR products from two gonadal cell populations. Cells from 12 to 14 day gonads were separated into two cell populations and analyzed by RT-PCR with the TN-AP primer pair and a primer pair specific for mRNA from the oncogene c-*kit*. Lane 1 is cell fraction 1, and lane 2 is cell fraction 2 amplified with the TN-AP primer pair; lane 4 is cell fraction 1, and lane 5 is cell fraction 2 amplified with the c-*kit* primer pair; lane 3 is empty.

histochemical and immunohistochemical studies suggest that AP protein is restricted to the ICM of late blastocysts (Ziomek *et al.* 1990).

The second AP gene transcribed and translated by mouse embryos is the one encoding the TN-AP isozyme. TN-AP is the major isozyme transcribed by 7.5, 8.5 and 9.5 day embryos and also is expressed by PGC, EC cells and placenta. At the level of transcription, it constitutes less than 20 % of the total AP mRNA during preimplantation development. The relatively low abundance of TN-AP mRNA compared to E-AP mRNA probably accounts for failure to detect this gene product at the enzymatic level in previous studies.

TN-AP and E-AP mRNAs are transcribed in adult mouse testis. A study with rat testis suggested that only the TN-AP isozyme was synthesized (Kornblatt *et al.* 1983); however, the general consensus from human studies is that both the TN-AP and GC-AP isozymes are active in adult testis (Chang *et al.* 1980; Goldstein *et al.* 1982; Millán *et al.* 1982; Hustin *et al.* 1987; Millán and Manes, 1988). We found that E-AP mRNA is only slightly less abundant than the TN-AP isozyme (Table 1), while less than 5 % of total AP activity in human testicular extracts has been found to be due to GC-AP (Chang *et al.* 1980). We do not know which testicular cell types express each isozyme in the mouse. Our results show that mouse testis expresses TN-AP and E-AP, but that mouse PGC express only the TN-AP isozyme.

Table 2. *Summary of AP gene expression in mouse embryos and tissues*

	E-AP	TN-AP	I-AP
Embryonic cells			
Unfertilized eggs	−	+/−	−
2-cell stage	+	+	−
8-cell stage	+	+	−
Blastocyst	+	+	−
7.5 day embryo	−	+	−
8.5 day embryo	−	+	−
9.5 day embryo	−	+	−
PGC	−	+	−
Nulli SCC1 EC	−	+	−
Adult tissues			
Testis	+	+	−
Liver	−	+	−
Intestine	+	+/−	+
Thymus	+	+	+

−, no detection by RT-PCR; +/−, occasional detection by RT-PCR; +, presence of RT-PCR products.

The third isozyme examined was predominantly transcribed in the intestine. I-AP mRNA was not detected in preimplantation embryos, testis or PGC. It was found, however, in thymus, along with mRNAs for TN-AP and E-AP (data not shown). It is interesting that E-AP mRNA also was found in intestine. Because the mRNA was isolated from whole small intestine (duodenum and upper jejunum) of adult mice, we probably are not detecting the fetal form of the intestinal isozyme (Vockley *et al.* 1984), and there are other reports of two AP forms in adult intestine (Garattini *et al.* 1985; Sussman *et al.* 1986).

E-AP and TN-AP are transcribed and translated by early mouse embryos: E-AP is the predominant isozyme of preimplantation embryos, and TN-AP is the major isozyme of 7.5 to 14 day embryos. We do not know when during early postimplantation development the switch in isozyme predominance occurs, or whether cells ever co-express the two isozymes. However, since the sequences of the two isozymes now are known, and either cDNA or genomic clones are available, it should be possible to examine the role of each AP isozyme in preimplantation development.

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