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Intron requirement for expression of the human purine nucleoside phosphorylase gene

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ABSTRACT

Abbreviated purine nucleoside phosphorylase (PNP) genes were engineered to determine the effect of introns on human PNP gene expression. PNP minigenes containing the first intron (complete or shortened from 2.9 kb down to 855 bp), the first two introns or all five PNP introns resulted in substantial human PNP isozyme expression after transient transfection of murine NIH 3T3 cells. Low level human PNP activity was observed after transfection with a PNP minigene containing the last three introns. An intronless PNP minigene construct containing the PNP cDNA fused to genomic flanking sequences resulted in undetectable human PNP activity. Heterogeneous, stable NIH 3T3 transfectants of intron-containing PNP minigenes (verified by Southern analysis), expressed high levels of PNP activity and contained appropriately processed 1.7 kb message visualized by northern analysis. Stable transfectants of the intronless PNP minigene (40–45 copies per haploid genome) contained no detectable human PNP isozyme or mRNA. Insertion of the 855 bp shortened intron 1 sequence in either orientation upstream or downstream of a chimeric PNP promoter-bacterial chloramphenicol acetyltransferase (CAT) gene resulted in a several-fold increase in CAT expression in comparison with the parental PNP-CAT construct. We conclude that human PNP gene expression at the mRNA and protein level is dependent on the presence of intronic sequences and that the level of PNP expression varies directly with the number of introns included. The disproportionately greatest effect of intron 1 can be explained by the presence of an enhancer-like element retained in the shortened 855 bp intron 1 sequence.

INTRODUCTION

In most eukaryotic genes, the coding sequence is interrupted by introns, which are included in initially transcribed RNA but are eventually removed by a precise splicing mechanism in the nucleus during the formation of mature messenger RNA (1). It has been suggested that introns might play a role in chromatin structure and its relationship to gene function (2), as well as contribute to evolutionary processes through 'exon shuffling' (3). Introns have been shown to regulate gene expression both transcriptionally and post-transcriptionally, sometimes through alternative splicing (4). Transcriptional regulatory elements have been identified in the introns of several genes (5–10), and many genes have been identified which are functionally impaired with introns removed (11–12).

The human purine nucleoside phosphorylase (PNP) gene, approximately 7 kb in length, is interrupted by 5 introns of varying sizes (13–14). The PNP gene product is an enzyme (purine-nucleoside orthophosphate ribosyltransferase; EC 2.4.2.1) which catalyzes the conversion of inosine and guanosine pentosides to the free nucleobase and pentose-1-phosphate, and is ubiquitously expressed in mammalian cells and tissues (15). The importance of PNP expression is evident in humans where it is observed that PNP-deficient individuals develop T-cell immunodeficiency (16), an autosomal recessive disease which is fatal during the first few years of life due to overwhelming infection. PNP deficiency is considered a prototype disease for human gene therapy (17) because its symptoms are largely restricted to the lympho-hematopoietic system (16).

Although maintenance of sufficient PNP activity *in vivo* is crucial to normal immunodevelopment, little is known about the regulation of PNP gene expression. We have been interested in characterizing regulatory elements in the human PNP gene which might be important to include in vectors designed for PNP expression after retroviral-mediated gene transfer into lympho-hematopoietic cells (18). In initial studies, we characterized the promoter region of the human PNP gene (19). Recently, it has been reported that in transgenic mice the presence of introns can have a marked effect on expression of a gene product (20–21). Retroviral-mediated introduction of human β-globin genes complete with introns into murine hematopoietic tissues has also been demonstrated to result in erythroid-specific expression (22–25). The relatively small size of the PNP gene makes it conducive to genetic manipulation, and here we report the

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construction of a series of PNP minigenes designed to determine the role of introns on PNP gene expression after transfection into cultured mammalian cells. Human PNP gene expression was evident by both enzyme and northern analyses in transfectants of all intron-containing PNP-minigene constructs, but was not detected in transfectants of an intronless PNP minigene construct. We also identified the presence of an enhancer-like element in the first intron of the PNP gene. The results indicate that introns are necessary for the generation of mature, human PNP message in transfected murine cell populations and that intron 1 sequences contribute to the regulation of transcription.

MATERIALS AND METHODS

DNA manipulations

Plasmids were maintained in *Escherichia coli* K-12 strain 294, and were extracted and purified as previously described (19). Plasmids used for transient transfection analyses were banded twice on CsCl-ethidium bromide gradients. Restriction enzymes were from New England Biolabs. Bacterial alkaline phosphatase, T4 DNA ligase and the Klenow fragment of DNA polymerase I were obtained from BRL. *Taq* DNA polymerase (Perkin-Elmer/Cetus) was used for the polymerase chain reaction (PCR).

Mammalian cell culture and gene transfer

Mouse NIH 3T3 tk^- (lacking thymidine kinase) cells (26) were routinely cultivated at 37°C and 5% CO_2 in Dulbecco-modified Eagle medium (DMEM, Gibco)/10% newborn calf serum, 2 mM glutamine, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. DNA-calcium phosphate coprecipitate-mediated transfections were as previously described (27–28). Briefly, 5×10^5 cells were subcultured one day prior to transfection and then exposed to precipitate overnight. In transient transfection experiments with PNP constructs, the molar equivalent of 10 μg of pPNPi1-5 was transfected unless otherwise specified. For CAT plasmids, the molar equivalent of 10 μg of pCAT(An) (29) was transfected. Ten μg of pHSV-106, a herpes simplex virus thymidine kinase expression plasmid (30) (BRL), was co-transfected to standardize for variation in gene transfer efficiency in all transient transfection experiments (31). For stable transfections, PNP minigene plasmid DNA was digested to completion with *Sal*I and then co-precipitated at a 2.5-fold molar excess with 1 μg of pFR400, a plasmid designed for expression of the murine arg22 variant, methotrexate-resistant dihydrofolate reductase (DHFR) (32). All transfected cells were shocked with 15% glycerol (33) in phosphate-buffered saline and, after a 2-day recovery period, either subcultured into DMEM containing 0.1 μM methotrexate (for establishment of stable transfectants) or harvested by trypsinization for enzyme analysis. Methotrexate-resistant colonies were harvested after two weeks, pooled and expanded in culture for further characterization. Extracts of PNP minigene-transfected cells were prepared by freeze-thawing 3 times in 80 μl 50 mM Tris pH 7.5. Crude cytoplasmic extracts were collected after centrifugation at 16,000$\times$g for 15 min (4°C) and desalted by Bio-gel P-10 (200–400 mesh; Bio-Rad) centrifugal column chromatography in 10 mM Tris pH 7.5. CAT plasmid-transfected cells were lysed by 3 cycles of freeze-thawing in 220 μl 50 mM Tris pH 7.8 and crude cytoplasmic extracts collected after centrifugation as previously described (19).

Table 1. PNP activity and integrant copy number in stable PNP minigene transfectants

Cell Line	PNP activity [a]	Copy Number [b]
HeLa	133 ± 1	
3T3	44 ± 3	
pPNPi0 #1	52 ± 2	40–45
pPNPi0 #2	59 ± 1	10–15
pPNPi1 #1	165 ± 48	1
pPNPi3-5 #1	471 ± 77	35–40
pPNPi3-5 #2	187 ± 21	10
pPNPi1-5 #1	204 ± 41	2
pPNPi1-5 #2	128 ± 23	4

[a] Pmol min^{-1} μg^{-1}; Values are expressed as the mean ± S.D. for 2–3 different extract preparations.

[b] Copy numbers per haploid genome, measured by videodensitometric analysis of Southern hybridization signals in Fig. 3B.

PNP isozyme analysis

Desalted cell extracts of transient or stable transfectants (15 μl) were subjected to isoelectric focusing between pH 4 and 6.5, followed by histochemical staining of the polyacrylamide gel for PNP activity as previously described (34). Human PNP activity, identified as material which focused at pI = 6.1, was quantitated by videodensitometry (35).

Enzyme assays

PNP activity was determined in crude cell extracts using a continuous assay at 290 nm (36) on a Beckman DU-50 spectrophotometer. Automated readings were used to compute initial velocities. Chloramphenicol acetyltransferase (CAT) activity was assayed by the method of Nordeen *et al.* using [^{3}H]-acetate as substrate (37) as described (19). Thymidine kinase (TK) activity was determined using [*methyl*-^{3}H]thymidine as substrate, monitoring the accumulation of [*methyl*-^{3}H]TMP by binding to polyethyleneimine-impregnated cellulose (31) and the resulting value used to normalize transiently expressed PNP or CAT activities. Protein was determined by the Coomassie brilliant blue microassay as formulated by BioRad using bovine serum albumin as standard.

Blot hybridization analysis

For Southern blotting (38) DNA was extracted as previously described (39) with the addition of RNase treatment, phenol-chloroform extraction and ethanol precipitation. Genomic DNA samples were digested with restriction endonucleases, subjected to electrophoresis in 0.7% agarose gels, and blotted onto Nytran according to the manufacturer's recommendation (Schleicher and Schuell). Poly(A)$^+$ RNA for Northern blots was extracted from cells using the FastTrack 3.0 kit (InVitrogen). RNA ladder (10 μg; 0.24–9.5 kb; BRL) and poly(A)$^+$ RNA samples (1 μg) were fractionated by electrophoresis in formaldehyde gels (40) and then blotted onto Nytran (Schleicher and Schuell) according to the manufacturer's instructions. All blots were prehybridized in 6$\times$SSPE, 10$\times$Denhardt's reagent, 1% SDS, and 150 μg ml^{-1} low molecular weight denatured DNA (plus 5 μg ml^{-1} yeast tRNA for Northern blots) for 1–2 hr or overnight at 42°C. Hybridizations were conducted overnight at 42°C in prehybridization solution with Denhardt's reagent replaced by 50% formamide and 10% dextran sulfate. Two probes radiolabelled by random priming (41) were used (Fig. 3A); a 1.5 kb *Ava*I fragment isolated from pPNPi0, or a 356 bp PCR product (30 cycles, 1 min 94°C, 2 min 58°C, 1 min 72°C)

generated from pPNPi1 with primers 5′-CCGGGGCAGAAA-GGTTTAGGGC-3′ (sense −174 to −153 bp upstream of the PNP transcriptional start site) and 5′-GTCACAGCACC-TGCCCCT-3′ (antisense, bases 35 to 52 in intron 1). Filters were washed twice at room temperature for 15 min in 6×SSPE/0.5% SDS, 4 times at 60°C for 30 min in 0.2×SSPE/0.5% SDS and then exposed to X-ray film for 1−4 days at −70°C using an intensifying screen. Autoradiographic signals were quantitated by videodensitometry (35).

RESULTS

Transient expression of human PNP minigenes in mouse NIH 3T3 cells

Plasmids were constructed to contain the human PNP coding sequence along with various complements of introns using human PNP genomic clones (13) and cDNA (13,42) as starting material (Fig. 1). To initially determine the effect of introns on human PNP gene expression, the different PNP minigene constructs shown in Fig. 1 were transfected into mouse NIH 3T3 cells and cell extracts assayed for human PNP activity by isozyme analysis as described in Materials and Methods. Representative results are shown in Fig. 2. As previously observed (34) the human PNP expressed transiently in mouse cells consisted primarily of the homotrimer (pI = 6.1) rather than heterotrimers containing both human and mouse PNP subunits. Human PNP was not detected in extracts of pPNPi0-transfected cells, but was detected at various levels in extracts of cells transfected with all of the other PNP minigene constructs, indicating that introns were necessary for transient expression of human PNP in these cells. To date, we have observed no detectable human PNP in pPNPi0 transfected cells in a total of 8 experiments. Several lines of evidence (see Discussion) indicated that the PNP coding sequence was intact in pPNPi0, and that the lack of PNP expression observed in pPNPi0 transfectants was not due to inadvertent disruption of the PNP coding sequence in pPNPi0 (14). We also investigated the role of a 10 bp direct repeat in the 5′ untranslated region (UTR) of PNP genomic sequences which was missing in the PNP cDNA clone (and in pPNPi0), but addition of this 10 bp repeat sequence to the 5′ UTR of pPNPi0 did not result in detectable human PNP expression (data not shown).

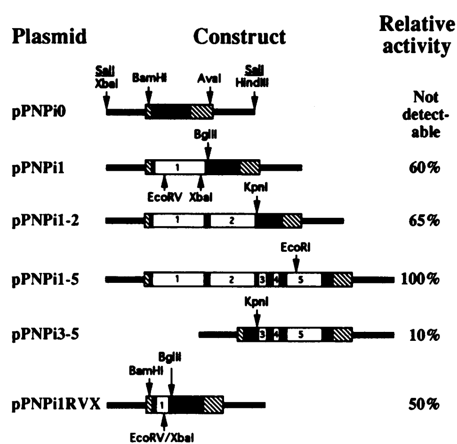

Fig. 1. Human PNP abbreviated gene constructs and summary of transient transfection results. Exons are illustrated by closed boxes and introns (numbered) by open boxes. Untranslated regions are hatched. Key restriction sites used for construction are indicated. All engineered PNP genes were constructed as *Sal*I cassettes inserted into a pUC19 vector (67) (BRL) modified to contain a *Hin*dIII-*Xba*I polycloning region at the 5′ end and a *Hin*dIII-*Sal*I-*Hin*dIII polycloning region at the 3′ end. All constructs contained a 5′ 2.2 kb *Xba*I-*Bam*HI fragment including the human PNP promoter (19) and a 3′ 1.4 kb *Ava*I-*Hin*dIII fragment containing the putative polyadenylation signal for the human PNP message (19, Jonsson, J.J. and McIvor, R.S., unpublished observations). The human PNP cDNA coding sequence (13,42), isolated from pEPD (34), was inserted between the 5′ and 3′ genomic flanks between *Bam*HI and *Ava*I to form pPNPi0. pPNPi1 was constructed by fusing the cDNA sequence to the 5′ flank at a *Bgl*II site located in exon 2, thus including the first intron along with the 5′ flanking sequence. pPNPi1-2 was similarly constructed using a *Kpn*I site located in exon 3, thus including the first two introns along with 5′ flanking sequence. pPNPi3-5 was constructed by fusing the cDNA sequence to the 3′ flank at the *Kpn*I site in exon 3, thus including introns 3, 4 and 5 along with the 3′ flanking sequence. The complete human PNP gene, which was not obtained on a single λ phage clone (13), was reconstructed in pPNPi1-5 by fusing overlapping PNP genomic clones (λ2 and λ4) (13) at an *Eco*RI site at the 5′ end of intron 5. pPNPi1RVX was constructed by removing 2 kb of intron 1 sequence from pPNPi1 between *Xba*I (Klenow filled) and *Eco*RV sites to leave an 855 bp shortened intron 1 sequence. Relative levels of transient human PNP expression in NIH 3T3 tk^- fibroblasts (example in Fig. 2) are shown on the right. Results are expressed as a percentage of the activity observed for pPNPi1-5 transfected cells after normalization for efficiency of gene transfer. Each value represents the average of three experiments.

Human PNP transient expression (Fig. 2) was quantitated by videodensitometry and the results are summarized in Fig. 1. Transfection with PNP minigenes containing either the first intron or the first two introns resulted in similar levels of human PNP activity. Transfection with pPNPi1-5, the complete PNP gene, resulted in a signal about 1.5 to 2-fold higher than with pPNPi1. Interestingly, transfection with pPNPi3-5, containing the last three introns, resulted in a human PNP activity level only 15% that of pPNPi1, indicating that not all introns contributed equally to increased human PNP expression after transfection into NIH 3T3 cells. Internal deletion of 2.0 kb from intron 1 (pPNPi1RVX) did

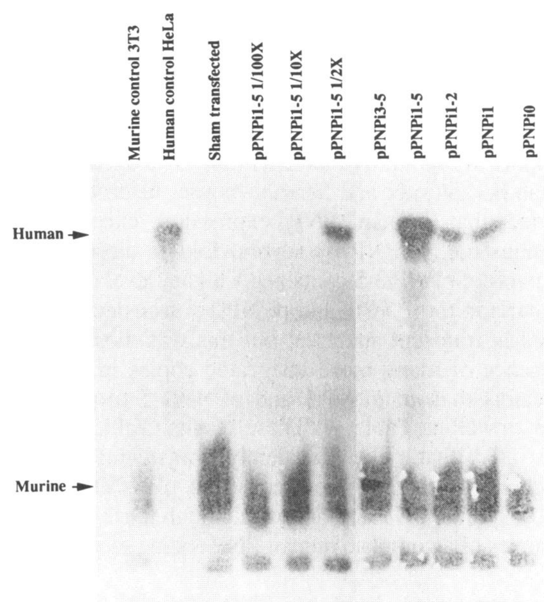

Fig. 2. Example of a transient human PNP expression experiment. NIH 3T3 fibroblasts were transfected with different PNP minigene constructs and assayed for human PNP expression as described in Materials and Methods. Lesser amounts of pPNPi1-5 (0.1 to 5 μg) as indicated were transfected as a linearity check for the assay. The sham control was transfected with 10 μg of calf thymus DNA. The locations of mouse (pI = 5.1) and human (pI = 6.1) homotrimers are indicated.

not significantly affect the level of human PNP expression in comparison with pPNPi1, indicating the presence of intron 1 sequences contributing to PNP expression in the remaining 855 bp.

Expression of human PNP activity in stable PNP minigene transfectants

In order to obtain enriched cell populations and thus provide a stronger signal for subsequent molecular analyses, heterogeneous stable transfectants were established for four of the PNP minigene constructs depicted in Fig. 1 (pPNPi0, pPNPi1, pPNPi1-5 and pPNPi3-5). Heterogeneous transfectant populations representing many colonies were prepared (see Materials and Methods) to minimize potential effects of flanking chromosomal sequences on expression of integrated PNP minigenes in more homogeneous populations.

Southern analysis was conducted to verify co-integration of PNP minigenes into drug-resistant transfectants and to quantitate integrant copy number. Integration of pPNPi0, pPNPi3-5, pPNPi1 and pPNPi1-5 was evident by the presence of PNP-hybridizing *Kpn*I fragments co-migrating with plasmid controls (Fig. 3B). However, videodensitometry of PNP-hybridizing signals demonstrated significant variation in integrant copy number, as summarized in Table 1. We also verified that pPNPi0 integrants contained complete coding sequences by digesting with *Ava*I, which cuts in the promoter and in the 3′ UTR releasing a PNP-hybridizing fragment of 1.5 kb present in 10−45 copies in these two cell populations (Fig. 3C).

Specific PNP enzyme activities were determined in cell extracts of stable PNP minigene transfectants (Table 1). Stable pPNPi0 transfectants contained no additional PNP activity over what was observed in the mouse NIH 3T3 control. Stable transfectants of the intron-containing constructs pPNPi1, pPNPi3-5 and pPNPi1-5 contained 3 to 10 times as much PNP activity as the parental NIH 3T3 cells. These extracts contained, in addition to homotrimers, heterotrimers consisting of different combinations of mouse and human PNP subunits identified by isozyme analysis (Fig. 4), consistent with previous results from stable transfectants which expressed the human PNP cDNA using heterologous transcriptional regulatory elements (34). The exclusive presence of human homotrimer and human$_2$-mouse$_1$ heterotrimer (Fig. 4) confirmed that human PNP expression exceeded that of endogenous mouse PNP by several-fold in these cells. Stable transfectants of pPNPi3-5 contained a higher level of PNP activity in comparison to pPNPi1 and pPNPi1-5 transfectants than that observed in transient analyses, but this was most likely due to the presence of many more integrated copies in the pPNPi3-5 transfectants than in pPNPi1 and pPNPi1-5 transfectants (see Fig. 3B as well as Table 1). These results (Table 1 and Fig. 4) demonstrated that, like transiently transfected cells, stable transfectants of the intronless construct pPNPi0 expressed no detectable human PNP activity even though these cells contained many copies of a complete human PNP coding sequence (Fig. 3).

PNP message levels in stable PNP minigene transfectants

Northern blot analysis was carried out to determine if the observed differences in human PNP enzyme activity expressed in NIH 3T3 cells transfected with different PNP minigenes was due to the accumulation of different message levels or aberrant splicing. The 356 bp PCR product used as probe contained only 11 bp of coding sequence (from exon 1) in common with the sequence of the mouse PNP message (43), and thus did not hybridize to the endogenous mouse PNP message under the conditions used.

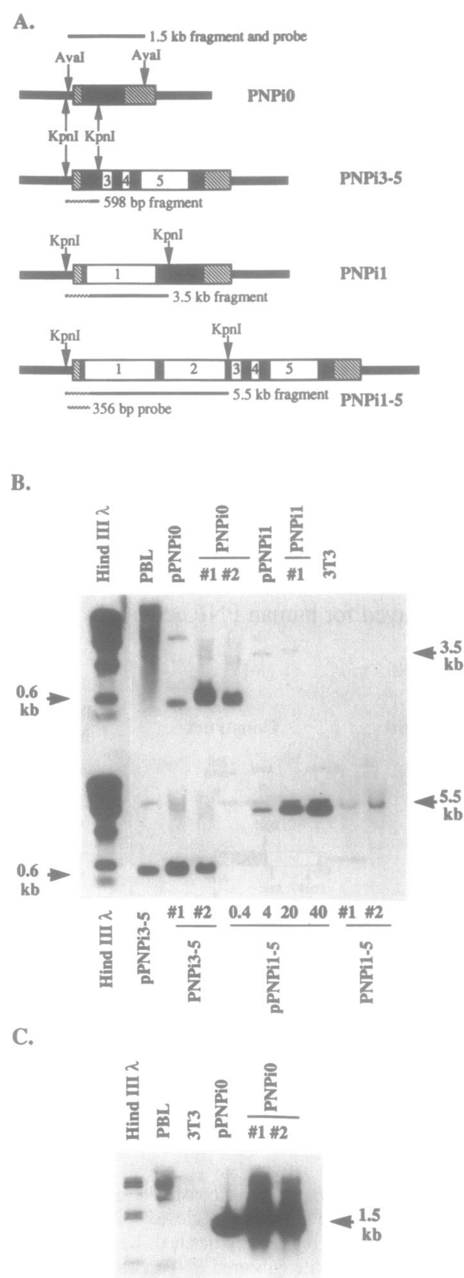

Fig. 3. Southern analysis of DNA extracted from stable NIH 3T3 transfectants of PNP minigenes. (A) PNP minigenes (as depicted in Fig. 1), expected restriction fragments, and probes utilized in the analysis. The probe used for *Kpn*I digests (part B) is hatched, as well as that portion of the *Kpn*I fragments hybridizing to the probe. (B) Southern blot of *Kpn*I-digested genomic DNA (10 μg) probed with a 356 bp PCR product from the 5′ end of the PNP gene generated as described in Materials and Methods. Locations of markers (λ *Hind*III) and expected fragment sizes for pPNPi0 (0.6 kb), pPNPi1 (3.5 kb), pPNPi1-5 (5.5 kb), and pPNPi3-5 (0.6 kb) integrants are indicated. *Kpn*I-digested human DNA and variable amount of pPNPi1-5 (equivalent to 0.4, 4, 20, and 40 copies per haploid genome) were included as controls. (C) Southern blot of *Ava*I-digested genomic DNA (10 μg) probed with a 1.5 kb *Ava* I fragment isolated from pPNPi0. Distinct pools of heterogeneous, stably transfected cells are numbered #1 and #2.

Stable pPNPi1-5 and pPNPi3-5 transfectants contained the highest levels of human PNP message (1.7 kb; Fig. 5), consistent

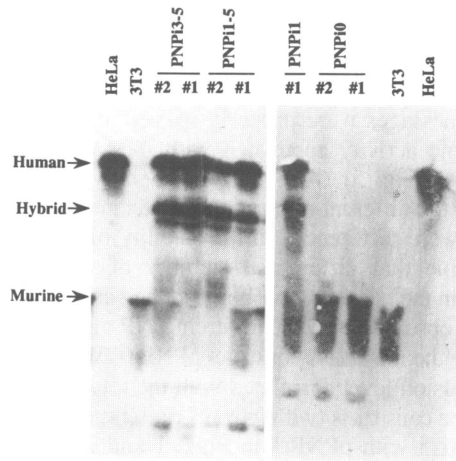

Fig. 4. PNP isozyme analysis of extracts from stable NIH 3T3 transfectants of PNP minigenes. Extracts from pPNPi0, pPNPi1, pPNPi1-5, and pPNPi3-5 are shown as well as control extracts from HeLa cells and the parental mouse NIH 3T3 cells. The locations of mouse (pI = 5.1) and human (pI = 6.1) homotrimers are indicated, as well as the positions of heterotrimers observed in some of the transfected cell extracts. Pools of heterogeneous stable transfectants are numbered # 1 or # 2. Data represent the results from two distinct but identically produced gels.

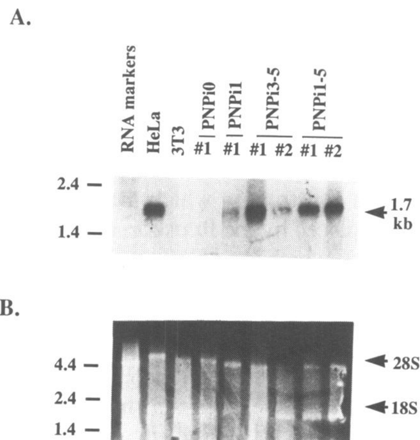

Fig. 5. Northern analysis of stable NIH 3T3 transfectants of PNP minigenes. (A) Northern blot of poly(A)$^+$ enriched RNA extracted from stable PNP minigene transfectants and probed for human PNP sequences with a 356 bp PCR product derived from 5½ end of the human PNP gene (see Materials and Methods). The locations of RNA markers as well as the human PNP message are indicated. PNP minigene transfectant populations are numbered # 1 or # 2. (B) The same RNA specimens as those depicted in part A were electrophoretically fractionated, stained with ethidium bromide, blotted and photographed under UV transillumination, demonstrating that similar amounts of RNA were loaded. The locations of RNA markers as well as 28S and 18S rRNAs are indicated.

with the higher level of specific PNP enzyme activity observed in these cells (Table 1). A lower level of PNP message was observed in pPNPi1 transfectant RNA, roughly proportional to the integrant copy number (Fig. 3, Table 1) and enzyme activity (Table 1) observed in these cells in comparison with pPNPi1-5 and pPNPi3-5 transfectants. Aberrant sizes of PNP-hybridizing material were not observed, indicating that messages from the different PNP minigenes were appropriately spliced in these cells. PNP message was not detected in pPNPi0 transfectant poly(A)$^+$ RNA, correlating with the absence of human PNP enzyme activity in NIH 3T3 cell transfectants of the intronless minigene pPNPi0. Since expression of the different PNP minigenes resulted in production of the same mature message, differences in accumulated levels of PNP message must have been associated with differences in message biogenesis prior to and including splicing, rather than differences in the stability of mature message. We conclude that the absence of human PNP activity in pPNPi0 transfectants resulted from the lack of mature PNP message generation.

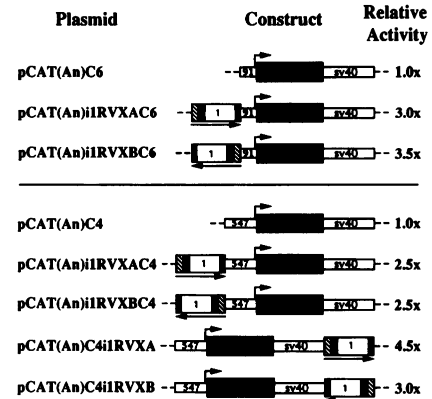

Fig. 6. An enhancer-like element in the first intron of the human PNP gene. A 1061 bp sequence extending from a *Bam*HI site in the 5′ UTR to a *Bgl*II site in exon 2 of pPNPi1RVX (Fig. 1) was subcloned into the *Bam*HI site in pUC9 to form pUC9i1RVX. A *Sal*I linker (NEB # 1148) was inserted into the Klenow enzyme filled *Bam*HI site, and then *Sal*I cassettes containing the shortened intron 1 were cloned in both orientations into a *Sal*I site upstream of a 91 bp PNP promoter in pCAT(An)C6 and a 547 bp PNP promoter in pCAT(An)C4 (19). Similarly a *Sac*I linker (NEB # 1044) was added to Klenow enzyme filled *Bam*HI and *Hin*dIII sites in pUC9i1RVX and *Sac*I cassettes containing the shortened intron 1 were cloned in both orientations into a *Sac*I site downstream of SV40 derived sequences containing the early region polyadenylation signal in pCAT(An)C4. Transient CAT levels in NIH 3T3 cells are expressed relative to the intronless parent constructs after normalizing for efficiency of gene transfer as described in Materials and Methods. Each value represents the average of three experiments.

An enhancer-like element in the first intron of the human PNP gene

PNP enzyme assays of NIH 3T3 cells transfected with the different PNP minigene constructs indicated that intron 1 had the greatest effect on PNP expression (Figs. 1, 2). To determine if intron 1 sequences contributed to the regulation of transcription initiation, the shortened intron 1 sequence retained in pPNPi1RVX (Fig. 1) was excised and inserted in both orientations upstream and downstream of a CAT transcription unit containing human PNP promoter sequences to regulate initiation of transcription (Fig. 6) (19). By inserting the intron 1 sequence upstream of a 91 bp PNP promoter in pCAT(An)C6 (19) a similar distance between potential regulatory sequences in intron 1 and the transcriptional start site was maintained since the PNP gene 5′ UTR and first exon are 130 bp long (14,19). We also inserted the intron 1 sequence upstream of a 547 bp PNP promoter in pCAT(An)C4 because we had determined in a separate experiment that the positive effect of intron 1 was retained when the 2.2 kb 5′ flanking sequence in pPNPi1RVX was truncated down to a 547 bp promoter (data not shown). Transfection of these plasmids into NIH 3T3 cells resulted in a 2.5- to 4.5-fold increase in CAT activity in comparison with

PNP-CAT plasmids not containing intron 1 inserts (Fig. 6). Increased expression was observed in both orientations for all three insert positions. These results demonstrated that an enhancer-like element (44) exists in the first intron of the human PNP gene, and that regulation of transcription initiation contributes to the intron-dependence of human PNP gene expression. However, this enhancer-like sequence was not capable of overcoming the apparent requirement for splicing, since no transient PNP expression was observed in NIH 3T3 cells transfected with plasmids containing the shortened first intron inserted in either orientation upstream of a 547 bp PNP promoter to regulate expression of the intronless PNP coding sequence.

DISCUSSION

In order to assess the role of introns in human PNP gene expression, we engineered abbreviated PNP genes containing different introns, transfected these PNP minigenes into mouse NIH 3T3 cells and assayed for human PNP activity. As observed for several other eukaryotic genes (10–12,45–46), much lower expression (in this case undetectable) was provided by an intronless minigene (pPNPi0) in transiently or stably transfected cells while substantial expression was observed in cells transfected with minigenes containing introns. These results indicate that intronic sequences either play a role in the rate of PNP transcription initiation, the stability of nascent RNA, or the efficiency of PNP message processing and delivery to the cytoplasm.

Intron-containing genes such as those encoding thymidine kinase (47), bovine growth hormone (48), and bean storage protein (49) have been found not to depend on the presence of introns for expression. However, the presence of introns has been shown to increase expression of SV40 sequences (46), mouse dihydrofolate reductase (11), ribosomal protein rpL32 (10) and several genes in maize cells (12). Transcriptional regulatory elements have been identified in intronic sequences (5–10), contributing to the control of tissue-specific expression of β-globin (7) and immunoglobulin (5,6) genes. For some systems the dependence of gene expression on the presence of introns is associated with the efficiency of splicing and generation of mature message from initial transcripts (10,46). Regulatory interactions between promoter and intronic sequence have been reported for immunoglobulin (50) and α1(I)collagen (8), and it has been suggested that intron-dependent expression can be promoter-specific, since intron dependence is lost when a heterologous promoter is used to regulate immunoglobulin gene transcription (51). Results presented in this paper demonstrate that intron-dependent PNP gene expression is at least partially due to the presence of a transcriptional regulatory element in the first intron (see Fig. 6). Although the relative role of splicing *per se* in PNP minigene expression has not been elucidated, the comparative level of PNP enzyme activity observed in transient transfections was very low for pPNPi3-5, containing the last three PNP introns (see Figs. 1 and 2). This could reflect a basal level of activity obtained when a PNP transcript is provided with access to the splicing machinery, while the increased level of expression observed for pPNPi1 results from the combination of increased efficiency of message processing as well as increased rate of transcription due to the presence of the enhancer. The inability of intron 1 enhancer-like sequences to bring about PNP expression when inserted upstream of an intronless PNP coding sequence further supports a basal requirement for splicing. The low level of PNP activity observed in pPNPi3-5 transient transfectants argues against the presence of a transcriptional regulatory element in the last three introns.

Northern analysis indicated that the initially transcribed PNP minigene messages were properly spliced and that the level of PNP enzyme activity correlated with message level in stable transfectants (Fig. 5). Although the identity of the processed message in the different minigene transfectants makes it unlikely that there were differences in the stability of mature message, it is possible that differential stability of initially generated transcripts in the nucleus contributed to the variable levels of PNP expression observed with the different PNP minigene constructs, particularly the intronless construct pPNPi0. We did observe that PNP expression level correlated with the total intron content of the minigene constructs (with intron 1 present; compare pPNPi1-2 and pPNPi1-5 with pPNPi1 in Figs. 1 and 2), consistent with the idea that more extensive association with the splicing machinery either protects the message from degradation or facilitates efficient delivery of the message from the nucleus to the cytoplasm.

The conclusion that introns are required for human PNP gene expression is dependent on the integrity of the PNP coding sequence in the intronless minigene pPNPi0, and we have made several observations which indicate that the PNP coding sequence in pPNPi0 was intact. First, pPNPi3-5, a minigene which was expressed after transfection into NIH 3T3 cells (Figs 1, 2, 4 and 5), was derived from pPNPi0, verifying that the coding sequence must be intact at least through the *Kpn*I site in exon 3. Secondly, a low level of PNP expression (presumably facilitated by the retrovirus enhancer) was observed when an intronless transcription unit derived from pPNPi0 was placed into a retrovirus and used to transduce PA317 cells (52). Finally, we have observed a low level of expression in NIH 3T3 cells transfected with a construct in which the PNP promoter region in pPNPi0 was replaced by the Rous sarcoma virus long terminal repeat (RSV-LTR), which is a very strong promoter in NIH 3T3 cells (53). The ability of these derivative constructs to provide expression of human PNP activity with the same isoelectric point (data not shown) as PNP extracted from HeLa cells indicated that the PNP coding sequence in pPNPi0 was intact.

High levels of PNP expression have been observed in fibroblasts infected with retroviral vectors in which human PNP expression is transcriptionally regulated by the Moloney murine leukemia virus (MoMLV) LTR (54,55). However, PNP expression at the RNA or protein level in fibroblasts has been low or undetectable when transcription of the PNP coding sequence is regulated from an internal heterologous promoter in retroviral vectors (18,54). This is consistent with the relatively low level of PNP expression observed for pPNPi0 or for PNP cDNA expression plasmids containing an SV40 early promoter or a mouse metallothionein I promoter transfected into Chinese hamster ovary or mouse L cells, respectively (34). We have also found expression of a murine PNP cDNA sequence to be undetectable using the SV40 early promoter (Nelson, D.M. and McIvor, R.S., unpublished observations) while substantial murine PNP was expressed using a MoMLV-LTR-regulated retroviral vector (43). Intron-dependent PNP expression is not limited to constructs containing the PNP promoter, since we observed a low level of transient PNP expression when the RSV-LTR was fused to an intronless PNP coding sequence but a substantial transient PNP signal when the same promoter was fused to a PNP minigene containing intron 1 (data not shown). Thus,

efficient expression of an intronless PNP coding sequence has been observed only when transcriptionally regulated by the MoMLV-LTR within the context of a full length retroviral vector genome, perhaps reflecting the different processing, cytoplasmic transfer and eventual fate of retroviral RNAs as opposed to cellular mRNAs (56). This presents a significant obstacle to the use of non-retroviral elements to regulate PNP expression after retroviral-mediated gene transfer.

Although PNP can be termed a 'housekeeping function', an enzyme of basic cellular metabolism ubiquitously expressed across a narrow range of activities in various tissues (57), there is evidence for differential expression of human PNP during thymic development (58), in peripheral lymphocytes (59), and in histochemical analyses of different tissues in experimental animals (60,61). Phytohemagglutinin induced T-cell transformation preferentially stimulates PNP synthesis in human T-lymphocytes (62) and phorbol ester stimulation increases PNP mRNA levels in human thymocytes (63) and leukemic cell lines (64). The identification of sequence elements which contribute to either regulated or constitutive PNP expression might be exploited for the purpose of engineering gene transfer systems optimized for expression under specially constrained conditions. Gene expression has been reported to be facilitated in transgenic animals by the presence of either natural (7,20) or heterologous (21) introns, and expression of genes in embryonal stem cells can require introns as well (65). Erythroid-specific expression of human β-globin sequences after retroviral-mediated gene transfer has been facilitated by the use of intact genes, including introns which contain regulatory sequences (22–25). The use of regulatory elements from a housekeeping gene (the murine DHFR promoter) has also been explored for extending the duration of observed expression in somatic cells (fibroblasts) transplanted into recipient animals (66). Use of intronic or flanking sequences from the PNP gene might similarly be beneficial for optimizing PNP expression after retroviral-mediated gene transfer into lympho-hematopoietic cells *in vivo*, a prospect which we are currently investigating.

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