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# Alternative polyadenylation produces two major transcripts of Alix

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## Abstract

The mammalian adaptor protein Alix participates in multiple cellular processes. Since mouse Alix cDNA detects two distinct transcripts of ~3.5 and ~7.0 kb in various mouse tissues, it is possible that there exist isoforms of Alix protein that perform varied biological functions. In this study, we first demonstrate that four different anti-Alix monoclonal antibodies immunoblot the single Alix protein in nine different mouse tissues. We then show that the two transcripts of 3.2 and 6.4 kb are widely expressed in various human tissues and cell lines. These two transcripts are generated from the same Alix gene localizing at 3p22.3 via alternative polyadenylation, thus containing an identical open reading frame. However, the 3.2-kb transcript is much more active in translation than the 6.4-kb transcript in a randomly selected cell line. These results eliminate the possibility that the two transcript variants encode different isoforms of Alix protein and suggest that alternative polyadenylation is one of the mechanisms controlling Alix protein expression.

### Keywords

ALG-2 interacting protein; Alix/AIP1/Hp95; gene structure; transcripts; alternative polyadenylation

## INTRODUCTION

Alix (ALG-2 interacting protein X), also called AIP1 or Hp95 [1-4], is a well-defined adaptor protein that has functions in diverse cellular processes, including apoptotic signaling [4-7], endo-lysosomal vesicular trafficking [8, 9], retroviral budding [10-13], actin cytoskeleton assembly [14], cytokinesis [15] and malignant transformation [4]. However, although anti-Alix antibodies detected only one Alix protein in multiple mammalian cell lines [3, 4, 14], 3.5-kb mouse Alix cDNA hybridized with two transcripts of ~3.5 and 7.0 kb from various mouse tissues [1, 2]. In the brain, the 7.0-kb transcript was even more abundant



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than the 3.5-kb transcript. These observations raise the issue of the identity and function of the extra 7.0-kb transcript. One intriguing possibility is that the 7.0-kb transcript produces an isoform of Alix protein, which performs a varied biological function. Obviously, clarifying these issues is a pre-requisite for profiling Alix expression in different normal and disease tissues by immunohistochemistry, RT-PCR or RNA expression arrays. The effort may also generate novel insights on the regulation of Alix function or expression.

Human genome and RNA transcripts are comprehensively sequenced in the last decade [16-18]. The results establish that the human genome contains ~25,000 unique genes [18] and that a large portion (>50%) of these genes produce multiple transcripts from the same pre-mRNA through choice of alternative sites for RNA splicing [19-23] or polyadenylation [24-28]. Accumulating evidence indicates that alternative splicing of the same pre-mRNA may produce transcripts with different biological functions [20], whereas alternative polyadenylation of the same pre-mRNA may produce transcripts that differ in stability [29-31] or translational efficiency [29, 32]. Thus, whenever alternative transcripts are generated from a unique gene, a formal possibility is raised that alternative RNA processing regulates the gene function or expression.

In this study, we immunoblotted total proteins extracted from nine mouse tissues with four different anti-Alix monoclonal antibodies and showed that only the currently understood Alix protein is detected. We then probed blots of polyA(+) RNAs isolated from various human tissues and cell lines with our human Alix cDNA and observed that the 3.2-kb human Alix cDNA hybridized with two transcripts of ~3.2 and 6.4 kb. Finally, we systematically characterize human Alix gene and RNA transcripts. These efforts lead to the resolution that the 6.4-kb transcript is generated by an alternative polyadenylation of the Alix pre-mRNA and that this transcript is inhibited in translation.

## MATERIALS AND METHODS

### Protein extraction from mouse tissues and immunoblotting

Mouse tissues were homogenized in 10 volumes of ice-cold RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 150 mM NaCl in 20 mM Tris-HCl, pH 7.4) supplemented with 1 mM PMSF and 1 μg/ml each of leupeptin, pepstatin A, and chymostatin (Roche Applied Science). After crude lysates were cleared by centrifugation at 10,000 x *g* for 10 min, protein concentrations of different samples were determined by using DC protein assay kit (Bio-Rad). Aliquots of 20 μg total proteins from different tissues were then resolved by 10% SDS-PAGE, transblotted onto nitrocellulose membrane and immunoblotted with 1A12, 1F7, 2H12 and 3A9 anti-Alix monoclonal antibody as described in our previous studies [14; manuscript in preparation].

### Cell culture and RNA isolation

Cell lines used in this study and the culture medium for each of them are listed in Table S1. In all cultures, medium was supplemented with 10% fetal bovine serum, and cells were cultured at 37°C with 5% $CO_2$ and >90% humidity. RNA transcription was inhibited by adding 25 μg/ml of 5,6-dichlorobenzimidazole (DRB, Sigma) to the culture medium [33].

Cells were collected at ~90% confluence for RNA isolation. Total RNA was isolated from cultured cells using Trizole® reagent (Invitrogen) according to manufacture's manual.

### Northern blotting hybridization

15 μg of RNA from each sample were separated by 1.5% agarose-gel electrophoresis [34]. Gel-separated RNAs were stained with ethidium bromide (EtBr), transferred onto Hybond-N membranes (RPN82N, Amersham Biosciences) and then hybridized with $^{32}$P-labeled cDNA probes. Alix cDNA was previously cloned in our lab [3] (GenBank accession no. **AF349951**). GAPDH cDNA was purchased from Invitrogen. The 3'-UTR probe of the 6.4-kb Alix mRNA was generated by polymerase-chain reaction (PCR) amplification of the corresponding region in BAC clone RP11-268B23 [GenBank accession no. **AC113168**, purchased from Children's Hospital Oakland Research Institute (CHORI) (http://chori.org/BACPAC/vectorframe.htm)], which contains the human Alix gene. PCR primers for this amplification were 5'-tgtgagatttgctgctgttgca-3' (forward) and 5'-gtggaaaaaggatgagagg-3' (reverse). $^{32}$P-labeled cDNA probes were generated by random priming method [35, 36]. Hybridization of RNA blots with $^{32}$P-labeled cDNA probes was carried out as previously described [37]. RNA blots containing poly(A)+ RNAs from various human tissues were purchased from Clontech. The relative abundance of the 3.2- and 6.4-kb Alix transcripts in each tissue was determined by analysis of scanned images with ImageQuant software, version 5.0 (Amersham Biosciences).

### Database search and sequence analyses

Human databases reference assembly, all assemblies, and the High Throughput Genomic Sequences (HTGS) at NCBI (National Center of Biotechnology of Information, http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9606) were blasted for human genetic loci that contain Alix cDNA matching sequences. Human RefSeq RNA, Non-RefSeq RNA, Build RNA and expression sequence tag (EST) databases at NCBI were blasted for human transcripts containing Alix RNA sequences. Exon and intron sequences of the human Alix gene at 3p22.3 were defined by aligning the 3.2-kb and 6.4-kb Alix mRNA sequences respectively with that of the BAC clone RP11-268B32.

### Reverse transcription and polymerase-chain reaction amplification

Poly(A)+ rich RNA isolation and reverse transcription were performed using The Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). PCR amplification was performed using the "Hot-start" approach as previously described [37]. PCR primers for a 5' region common to both the 3.2-and 6.4-kb transcripts were 5'-ctgacaaaatcaatcgtgcc-3'(forward) and 5'-ccaaagactgctgtactgac-3'(reverse). PCR primers for a unique 3'-UTR region of the 6.4-kb transcript were the same as those described in the Northern blotting section. PCR products were separated by 1.5% of agarose-gel electrophoresis along with DNA molecular weight standards purchased from Invitrogen and stained with EtBr.

### Polyribosome isolation

Monolayer cultures of IMR90 cells in five 150-mm plates were washed with PBS, and cells were trypsinized and pelleted by centrifugation. Cell pellets were then resuspended in 2-ml

lysis buffer consisting of 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM MgCl2, 140 mM sucrose, 2 mM PMSF (phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride, Sigma), 200 μg/ml heparin (Sigma), 1% Triton X-100 (Calbiochem), 0.5% sodium deoxycholic acid (Sigma), and 100 μg/ml cycloheximide (Sigma) and incubated on ice for 15 min. Crude cell lysates were centrifuged at 12,000 x *g* for 10 min at 4°C and supernants were collected. One ml of cleared cell lysates was laid on top of 3.5 ml 1 M sucrose in a 5-ml Thickwall polyallomer tube (Beckman Coulter Ultrocentrifuge, Fullerton, CA), which was then centrifuged at 234,000 x *g* for 100 min at 4°C. After supernatants were removed, polyribosomes pelleted at the bottom of the tube were collected for RNA isolation.

## RESULTS

### Multiple anti-Alix monoclonal antibodies detect one Alix protein in various mouse tissues

To determine whether the two Alix transcripts expressed in various mouse tissues produce two isoforms of Alix protein, we extracted total proteins from 9 different mouse tissues and immunoblotted 20 μg protein from each of the samples with 1A12, 1F7, 2H12 or 3A9 anti-Alix monoclonal antibody. These four antibodies were generated in our previous studies [14]. As recently determined by immunoblotting of GST-tagged Alix recombinant proteins with each of these four antibodies (manuscript in preparation), these four antibodies recognize at least three different regions in Alix (Figure 1A). We reasoned that if the extra transcript of 7.0 kb produces an isoform of Alix protein, this protein should be recognized by one or multiple of these anti-Alix antibodies. We observed, however, that in all 9 mouse tissues tested, only one polypeptide of ~90 kd is readily recognized by each of the four monoclonal antibodies although the level of this protein varied among different tissues, being the lowest in brain and highest in lung. Much longer exposures of the blots still did not reveal extra significant polypeptides that were recognized by any of these four antibodies (Figure 1B). These results make it unlikely that the extra transcript of 7.0 kb expressed in various mouse tissues produces an isoform of Alix protein.

### Human Alix cDNA detects two major transcripts of 3.2 and 6.4 kb in various human tissues

To determine whether human tissues also express both the expected Alix mRNA and an additional high molecular weight transcript that contains Alix mRNA sequence, we hybridized two poly(A)+ RNA blots from human tissues with our human Alix cDNA. The RNA blots, which were commercially made with normalized procedures, contained similar amounts of poly(A)+ RNA from 16 different human tissues. The human Alix cDNA was cloned by degenerate PCR and RACE of a human placenta cDNA library based on its sequence homology to Xp95, the Xenopus ortholog of Alix [3]. This Alix cDNA is ~3.2 kb, containing a 5'-untranslated region (UTR), an open reading frame and a 3'-UTR plus a poly(A) tail. Hybridization of the total RNA from HeLa cells detected one major transcript of ~3.5 kb [3]. As shown in Figure 2A, the Alix cDNA detected two transcripts of ~3.5 kb and ~7.0 kb in all of the tissues examined (Figure 2A), indicating that human tissues also express both the expected Alix mRNA and an extra high molecular weight transcript that contains Alix mRNA sequence. Since the actual molecular weights of these two transcripts were later defined to be ~3.2 kb and 6.4 kb, these two transcripts are called 3.2-kb transcript and 6.4-kb transcript, respectively. Figure 2B shows the ratio between the two transcripts in

each of the examined human tissues. While the two transcripts were of similar abundances in liver, kidney, spleen and thymus, the 3.2-kb transcript was clearly less abundant than the 6.4-kb transcript in brain and ovary and clearly more abundant than the 6.4-kb transcript in the remaining 10 tissues. The dramatically different ratios between the two transcripts in different tissues predict that production of the two transcripts is a regulated process.

### Human cell lines also express both the 3.2-kb Alix mRNA and the 6.4-kb transcript

Although our previous Northen hybridization of HeLa cell total RNA with our Alix cDNA detected only one transcript of ~3.5 kb, mouse Alix cDNA detected both a major transcript of ~3.5 kb and a lower abundant transcript of ~7.0 kb in two human glioma cell lines [5], suggesting that the 6.4-kb transcript is also expressed in human cell lines at a low abundance. To examine this possibility, we isolated total RNA from a variety of human cell lines and performed Northern blot hybridization with our Alix cDNA followed by a long exposure of the blots. Our initial hybridization of total RNA from HeLa and human fibroblast IMR90 cell lines showed that Alix cDNA detected not only the 3.2-kb Alix mRNA as anticipated, but also the 6.4-kb transcript at a much lower abundance in both cell lines (Figure 3A). In the following hybridization of total RNA from 10 different head/neck and lung cancer cell lines, Alix cDNA detected both the 3.2-kb and the 6.4-kb transcripts in seven cell lines examined (Fig. 3B). These results demonstrate that the 6.4-kb transcript is also expressed in human cells, although at a much lower abundance.

### The 6.4-kb transcript is an alternative product of the unique Alix gene at 3p22.3

To investigate the origin of the 6.4-kb transcript that contains Alix mRNA sequence, we first did a BLAST search of human genome databases with our Alix cDNA sequence to determine whether multiple genetic loci may produce transcripts that can be hybridized with our Alix cDNA. Our 3242-bp Alix cDNA encodes a 132-bp 5'-UTR, a 2604-bp open reading frame, and a 506-bp 3'-UTR plus a poly(A) tail [3]. The search identified BAC clones from three different chromosome loci, which contain sequences that match the entire or part of the Alix cDNA with >95% identity (Figure 4A). One genetic locus is at 3p22.3, which contains sequences that match the entire Alix cDNA with >99% identity. Two other identified genetic loci, 15q11.2 and 15q13.1, each contains sequences that match one third of the Alix cDNA with >95% identity. Since the 3p22.3 is the only genetic locus that contains DNA sequences matching the entire Alix cDNA, this locus contains the unique Alix gene, responsible for producing the 3.2-kb Alix mRNA. On the other hand, the partial sequence match of the other two genetic loci raises the possibility that these two genetic loci produce the 6.4-kb transcript. To explore this possibility, we searched NCBI human RNA databases for RNAs produced by these genetic loci. A 2090-base transcript (LOC283683, GenBank accession no. **XR 015696**) from 15q11.2 and a 1680-base transcript (LOC646278, GenBank accession no. **XR 016361**) from 15q13.1 were found. Although both transcripts contain a middle region of 1060-base that is >95% identical to residues 1189-2251 of the Alix mRNA (Figure 4B), their sizes are much smaller than the 6.4-kb transcript, making it unlikely that the 15q11.2 or 15q13.1 locus produces the 6.4-kb transcript hybridized by Alix cDNA.

Since the 6.4-kb transcript is unlikely to be produced from the 15q11.2 or 15q13.1 locus, we next examined the possibility that the 6.4-kb transcript is an alternatively processed product from the 3p22.3 locus. To this end, we searched human RNA databases for alternative mRNAs from 3p22.3 that contain the entire Alix coding sequence. The search identified three types of mRNAs, which differ only in untranslated regions (Figure 4C). The first type contains multiple entries of the 3.2-kb Alix mRNA by us (GenBank accession no. **AF349951**) and others (GenBank accession no. **Af151793**; GenBank accession no. **BC068454**). The second type is a 3.6-kb mRNA (GenBank accession no. **BC020066.1**) identified from analysis of more than 15,000 full-length human and mouse cDNA sequences [Mammalian Gene Collection (MGC)] by National Institutes of Health. This mRNA is 479-base longer than the 3.2-kb mRNA in 3'-UTR. The third type is a 6.4-kb mRNA (GenBank accession no. **NM 013374**), which was assembled by NCBI staff from three partial cDNAs. As compared to the 3.2-kb mRNA, this 6.4-kb mRNA is 533-base longer in 5'-UTR and 2698-base longer in 3'-UTR. Both of the extra sequences find complete matches from the 3p22.3 locus, confirming its origin from this genetic locus. These results lead us to hypothesize that the 6.4-kb transcript is an alternatively processed transcript from the 3p22.3 locus. To test this hypothesis, we designed a pair of primers that specifically amplify a region in the 3'-UTR of the 6.4-kb mRNA (Figure 4D) and amplified this region from a cDNA pool made from HeLa cell total RNA by PCR. The PCR generated a fragment of an expected length (Figure 4E), indicating that the 6.4-kb mRNA is indeed expressed in HeLa cells. In addition, we amplified this 3'-UTR fragment of the 6.4-kb mRNA from genomic DNA by PCR and hybridized the total RNA isolated from IMR90 and 2008 cells with either the PCR product or Alix cDNA under a stringency that did not allow hybridization of the PCR product with the 3.2-kb mRNA. While the Alix cDNA hybridized to both the 3.2- and 6.4-kb transcripts as expected, the PCR product hybridized only to a transcript that co-migrated with the 6.4-kb transcript detected by Alix cDNA (Figure 4F). These results prove the hypothesis that the 6.4-kb Alix mRNA in database is the 6.4-kb transcript detected by Alix cDNA in Northern hybridization and that the 6.4-kb transcript is an alternatively processed transcript from the 3p22.3 locus.

### The 6.4-kb Alix transcript is produced by alternative polyadenylation

Alternative splicing, alternative transcription initiation and alternative polyadenylation are the most common mechanisms that generate alternative transcripts from the same gene. To determine whether alternative splicing is responsible for producing the 6.4-kb mRNA from the 3p22.3 locus, we first aligned Alix genomic DNA sequence with the 6.4-kb Alix mRNA sequence and deduced exon and intron sequences. For this transcript, Alix gene is ~72 kb in length, consisting of 18 exons and 17 introns (Figure 5A, upper panel). The exact sequences of exons and introns in BAC clone 268B23 (RP11-268B23, GenBank accession no. **AC113618**) are described in Table S2. We then aligned the Alix genomic DNA with the 3.2-kb mRNA and determined whether the 3.2-kb Alix mRNA is produced from the same or different exons. Although the 3.2-kb Alix mRNA is half of the size of the 6.4-kb mRNA, it is produced from the same 18 exons as that producing the 6.4-kb mRNA (Figure 5A, lower panel), making it unlikely that the extra UTR sequences in the 6.4-kb mRNA are produced by alternative splicing of Alix pre-mRNA.

Since the 6.4-kb mRNA is unlikely to be produced by alternative splicing of Alix pre-mRNA, we next analyzed the DNA sequences in the first and last exons of the Alix gene to ascertain whether the 5'-UTR and 3'-UTR extra sequences in the 6.4-kb mRNA may be produced by alternative transcription initiation and alternative polyadenylation of the Alix pre-mRNA, respectively. While four TATA sequences were identified at 334-374 bp upstream of the 5' matching region for the 6.4-kb mRNA, no TATA sequence was found within the 533-bp sequence upstream of the 5' matching region for the 3.2-kb mRNA (data not shown). These findings argue against the possibility that the 6.4- and 3.2-kb mRNAs are produced from different transcription initiation sites. While the actual reason for the difference in the length of 5'-UTR sequence between the 3.2- and 6.4-kb mRNAs remains unclear, one possible explanation for the difference is in the completeness of 5'-end sequencing for the two mRNAs. Analysis of the last exon sequence revealed that it contains six copies of AATAAA (Figure 5B), which is a highly conserved polyadenylation signal (PAS) [29, 38, 39]. While the 3.2-kb mRNA ends at 11 residues after the 1$^{st}$ PAS, the 6.4-kb mRNA ends at 15 residues after the 6$^{th}$ PAS. In between, the 3.6-kb Alix mRNA ends at 16 residues after the 4$^{th}$ PAS (Figure 5C). These results indicate that the 6.4-kb Alix mRNA is generated by an alternative polyadenylation of Alix-pre-mRNA.

### The 6.4-kb transcript is not engaged in translation in a human cell line

To determine the functional significance of the extra UTR sequences in the 6.4-kb transcript, we first compared the stability of the 3.2-kb transcript with that of the 6.4-kb transcript in IMR90 and T80 cells during treatment with the transcription inhibitor 5,6-dichlorobenzimidazole [33]. The 3.2- and 6.4-kb transcripts slowly decayed at similar rates (data not shown), making it unlikely that the extra UTR sequences in the 6.4-kb transcript affect the RNA stability. Second, we determined association of the 3.2-kb and 6.4-kb transcripts with polyribosomes, which contain RNA active in translation. Total and polysomal RNAs were isolated from IMR90 cells as diagrammed in Figure 6A, and the two RNA samples were hybridized with Alix cDNA. While the Alix cDNA detected both the 3.2- and 6.4-kb transcripts in the total RNA, the same probe detected only the 3.2-kb Alix transcript in the polysomal RNA sample (Figure 6B), indicating that only the 3.2-kb transcript is active in translation. To further examine this issue, we amplified the total and polysomal RNAs with two pairs of primers by RT-PCR, a method much more sensitive than Northern blotting hybridization. As diagrammed in Figure 6C, one pair of primers were designed to amplify a common 5'-end region of 269-bp for both the 3.2-kb and 6.4-kb transcripts, and the other pair were designed to specifically amplify a 768-bp region in the 3'-UTR of the 6.4-kb transcript. We observed that while the first pair of primers clearly amplified a fragment of an expected length from both the total and polysomal RNAs, the second pair of PCR primers amplified a fragment of an expected length only from the total RNA (Figure 6D). These results support the conclusion that only the 3.2-kb transcript is active in translation in IM90 cells. Together, these results indicate that the extra UTR sequences in the 6.4-kb transcript have negative effects on translation at least in IM90 cells.

## DISCUSSION

Both mouse and human tissues express two major transcripts that can be readily hybridized with the mouse or human Alix cDNA obtained in different laboratories. While one of the transcripts, which we call Alix mRNA or the 3.2-kb transcript, matches the size of the probing Alix cDNA, the other transcript, which we call the extra transcript or the 6.4-kb transcript, doubles the size of the probing Alix cDNA. This consistent and distinct phenomenon raises three issues that need to be resolved before Alix expression in normal and disease tissues may be confidently measured by commonly utilized techniques of immunohistochemistry, RT-PCR or RNA expression array. The first issue is whether the extra transcript is produced from the same Alix gene by alternative RNA processing or a different gene that share long stretches of exon sequences with the Alix gene. The second issue is whether the extra transcript encodes a protein that is highly similar to Alix, ie, a variant form of Alix protein. The third issue is whether expression of this extra transcript may have regulatory functions. In this study, we demonstrate that the extra transcript is produced from the unique Alix gene localized at 3p22.3 through alternative polyadenylation. The extra transcript is identical to Alix mRNA in coding sequence, making it impossible that the extra transcript produces an Alix variant. However, the extra transcript is much less active in translation than Alix mRNA in a randomly selected cell line, indicating regulatory functions of expressing this RNA variant. Since the 6.4-kb transcript contains the entire Alix mRNA, neither RT-PCR nor hybridization-based RNA expression arrays may specifically measure the level of Alix mRNA in different tissues or cell lines. Thus Northen blot analysis should be a more appropriate method. On the other hand, because the extra transcript does not produce a variant form of Alix protein, the extra transcript should not create confusions in using immunohistochemistry or tissue arrays to determine Alix protein expression in different tissues or cell lines.

Polyadenylation of a pre-mRNA is catalyzed by a cleavage/polyadenylation machinery, consisting of a specificity component that recognizes the polyadenylation signal AAUAAA, a cleavage stimulatory factor that recognize U/UG-rich elements downstream of the cleavage site, an endonuclease, and a poly(A) polymerase [29, 39-41]. Once the specificity component binds to an AAUAAA site and the stimulation factor binds the downstream U/UG-rich element in 3’-UTR of a pre-mRNA, the endonuclease component cuts the RNA at 10-30 bp downstream of the AAUAAA, and the polymerase synthesizes the poly(A) tail of up to 200 bases at the cutting site of the RNA [39, 42]. When a pre-mRNAs contains multiple AAUAAA sequences within the 3’-UTR, utilization of each of the polyadenylation sites is determined by the strength of the interaction between the polyadenylation machinery and the polyadenylation signal and the downstream U/UG-elements [39-42]. The strength of the interaction can be modulated by transacting factors that bind to 3’-UTR [26, 43].

Alix pre-mRNA contains at least 6 polyadenylation signal in the 3’-UTR. While the 3.2- kb Alix RNA is polyadenylated at 11 bases after the 1$^{st}$ AAUAAA site, the 6.4-kb Alix RNA is polyadenylated at 15 bases after the 6$^{th}$ AAUAAA site despite the presence of five AAUAAA sites upstream. Since the ratio between these two transcripts dramatically varies among different tissues (Figure 1), it is unlikely that their relative production is only controlled by cis-acting elements. In contrast, it is conceivable that certain transacting

factors, which are regulated during cell differentiation, may inhibit the recognition of the other five more proximal polyadenylation signals or specifically promote the recognition of the 6th polyadenylation signal by the machinery and cause production of the 6.4-kb Alix RNA. Tissues specific regulation of polyadenylation has been reported in multiple previous studies [26, 32, 44-46].

In human, ~50% pre-mRNAs undergo alternative polyadenylation [28]. In many cases, alternative polyadenylation does not affect RNA's function or metabolism. However, there are also clear cases in which alternatively polyadenylated transcripts containing the same coding region differ dramatically in RNA stability or translational efficiency. For example, alternative polyadenylation produces two distinct cyclooxygenase-2 (COX-2) transcripts of 2.8- and 4.6-kb in a tissue-specific manner, and the 4.6-kb transcript is less stable than the 2.8-kb transcript [30]. On the other hand, alternatively polyadenylated transcripts for human apoptosis-induced gene hap product and histone H4 transcription regulator BZW1 (basic leucine zipper and W2 domains 1) differ in translation efficiency [32, 46]. In the case of Alix transcripts, no difference was observed in the RNA stability between the 3.2- and 6.4-kb transcripts when IMR90 and T80 cells were treated with a transcription inhibitor, arguing against the possibility that alternative polyadenylation regulates the stability of Alix transcripts. However, while the 3.2-kb transcript was found to associate with polyribosomes in IMR90 cells as expected, the 6.4-kb transcript was undetectable in the polysomal fraction of RNA, indicative of the inhibitory effects of the extra UTR-sequences in the 6.4-kb transcript on its association with the translational machinery in this cell line. Although these results may not necessarily indicate that the extra transcript cannot be translated under any physiological or pathological conditions, these results generate a solid hypothesis that alternative polyadenylation is one of the mechanisms that regulate Alix protein expression.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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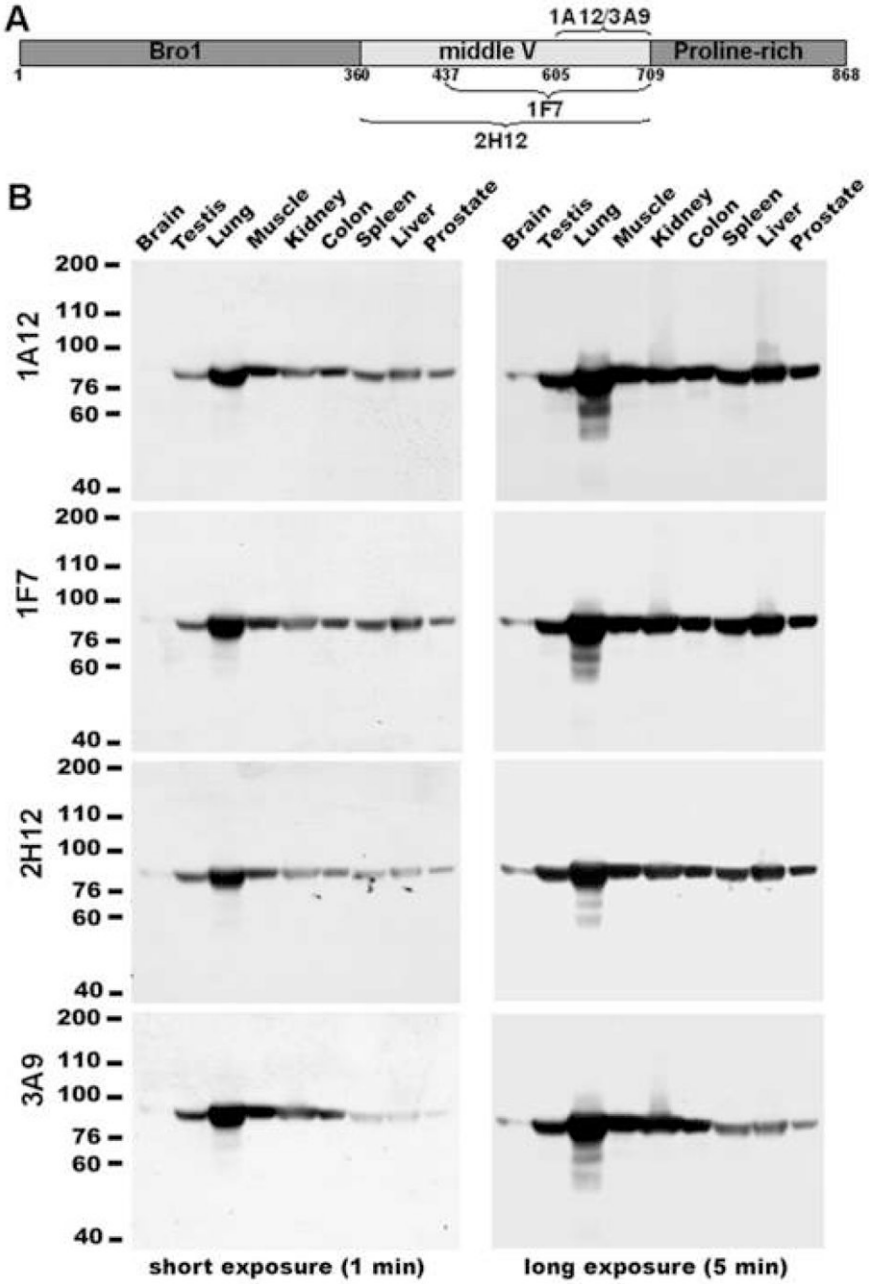


**Figure 1. Multiple anti-Alix monoclonal antibodies detect one Alix protein in various mouse tissues**

(A) Schematic illustration of recognition regions of four different anti-Alix monoclonal antibodies. (B) Total proteins from different mouse tissues were immunoblotted with each of the four anti-Alix antibodies, and the blots were exposed for 1 min (short exposure) and 5 min (long exposure).

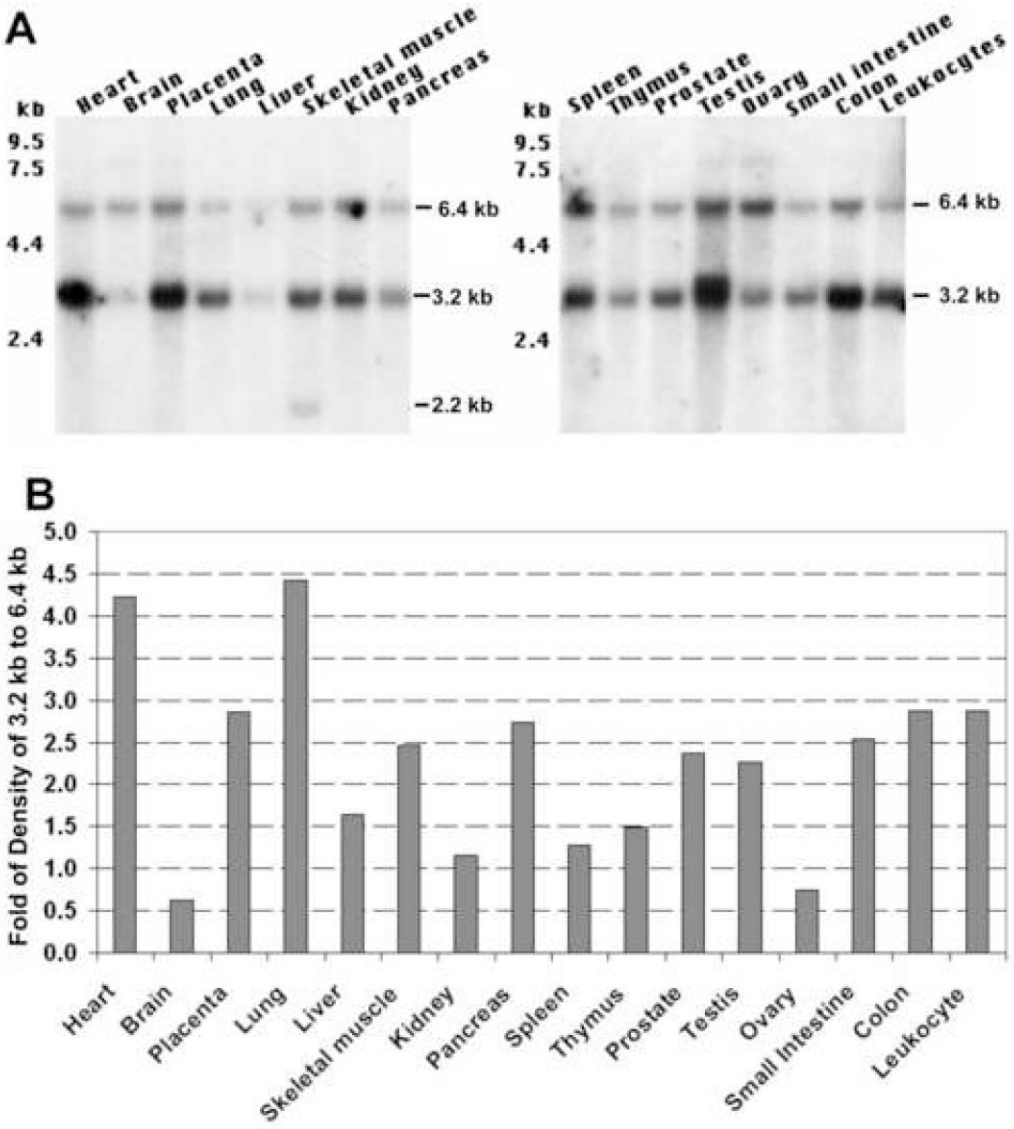


**Figure 2. Human Alix cDNA detects two major transcripts of 3.2 and 6.4 kb in various human tissues**

(A) Northern hybridization of poly(A)$^+$ RNA from 16 different human tissues with Alix cDNA. (B) The ratio of the 3.2-kb Alix transcript to the 6.4-kb Alix transcript in different human tissues in (A).

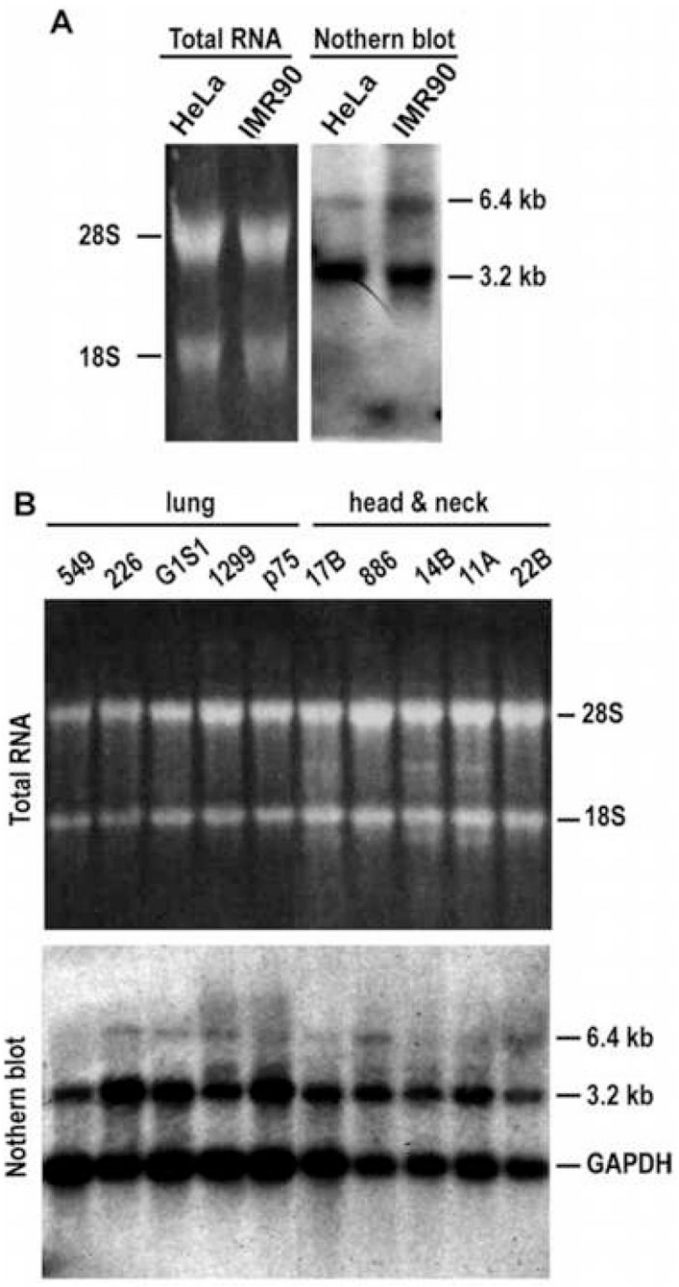


**Figure 3. Alix cDNA detects two transcripts in human cells**

(A) Northern hybridization of the total RNA extracted from IMR90 and HeLa cells with Alix cDNA. Left panel: EtBr staining of agarose gel-separated RNA. Right panel: autoradiography of hybridized RNA. (B) Northern hybridization of total RNA extracted from indicated head/neck and lung cancer cell lines with Alix cDNA and GAPDH cDNA. Upper panel: EtBr staining of agarose gel-separated RNA. Lower panel: autoradiography of hybridized RNA.

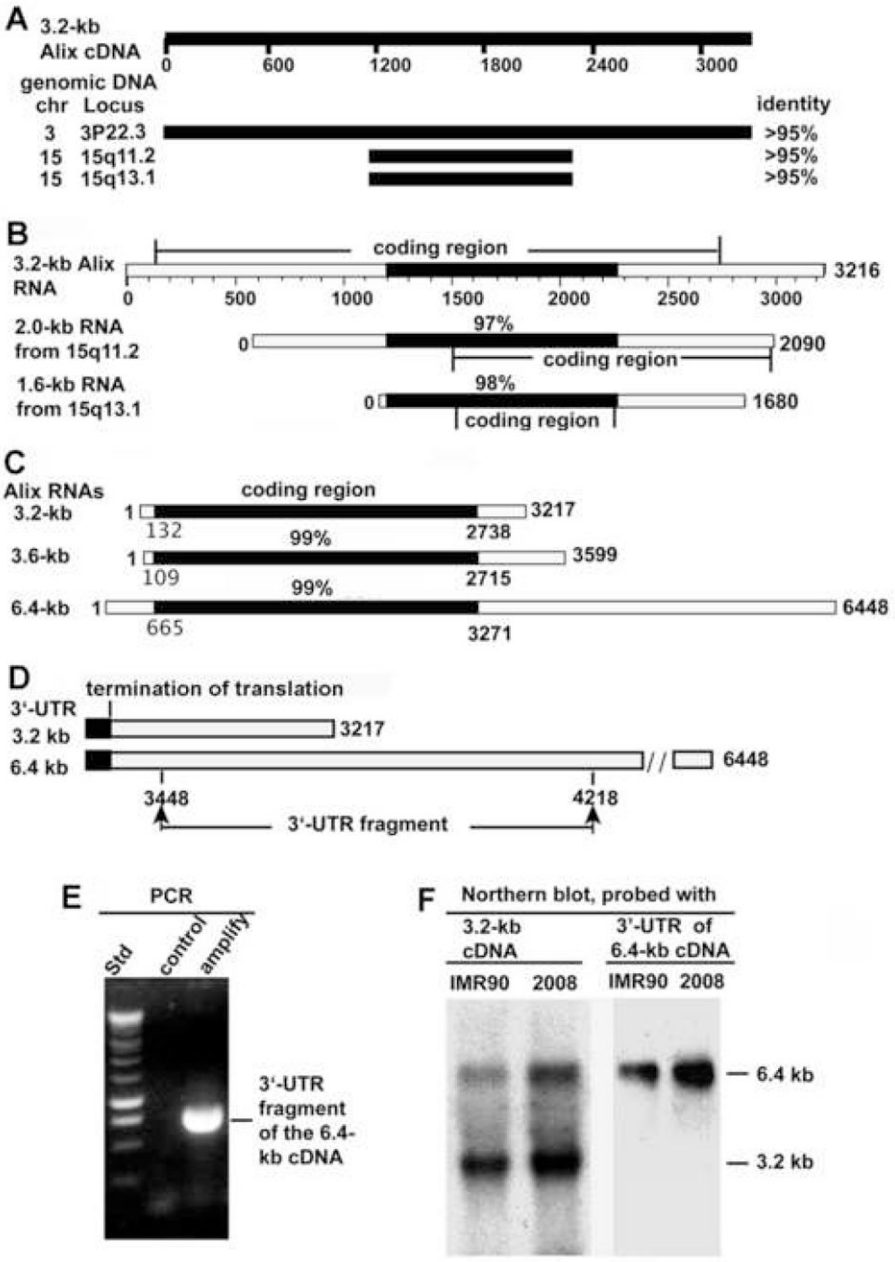


**Figure 4. The 6.4-kb transcript is an alternative transcript of the unique Alix gene at human 3p22.3**

(A) Schematic illustration of three genetic loci from two chromosomes (chr) that contain Alix matching sequences. (B) Schematic illustration of mRNAs from 15q11.2 and 15q13.1 and their alignment with the 3.2-kb Alix mRNA. Filled bar fragments indicate Alix matching regions. (C) Schematic illustration of three types of Ailx mRNAs produced from 3p22.3. Filled bar fragments indicate Alix coding region. (D) Schematic illustration of the region in 3'-UTR of the 6.4-kb Alix mRNA to be amplified by PCR. (E) HeLa cell cDNA was amplified for the region indicated in (D) by PCR, and products were separated by agarose gel electrophoresis and stained with EtBr. Control sample did not contain cDNA

template in the reaction. (F) Total RNAs from IMR90 and 2008 cell lines were hybridized with Alix cDNA (left panel) or the PCR-amplified region illustrated in (D).

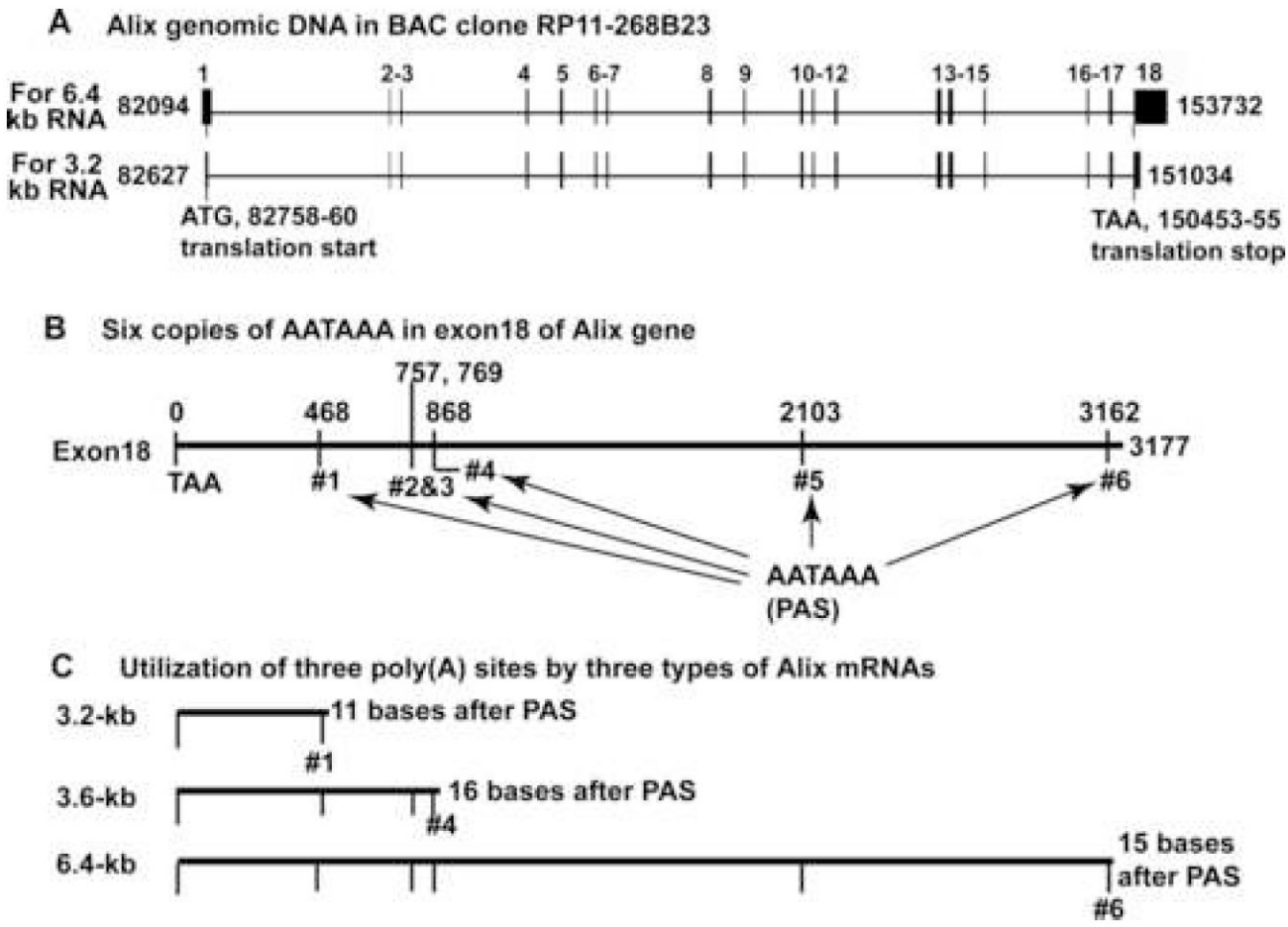


**Figure 5. Different Alix transcripts are produced by alternative polyadenylation**

(A) Schematic illustration of Alix introns and exons for the 6.4-kb (upper panel) and 3.2-kb (lower panel) Alix RNAs. Exons are shown as vertical lines and indicated by numbers and introns are indicated by the horizontal line between exons. (B) Schematic illustration of six polyadenylation signals (PAS) in the 3'-UTR of Alix pre-mRNA. (C) PASs chosen by the 3.2-kb, 3.6-kb and 6.4-kb Alix mRNAs.

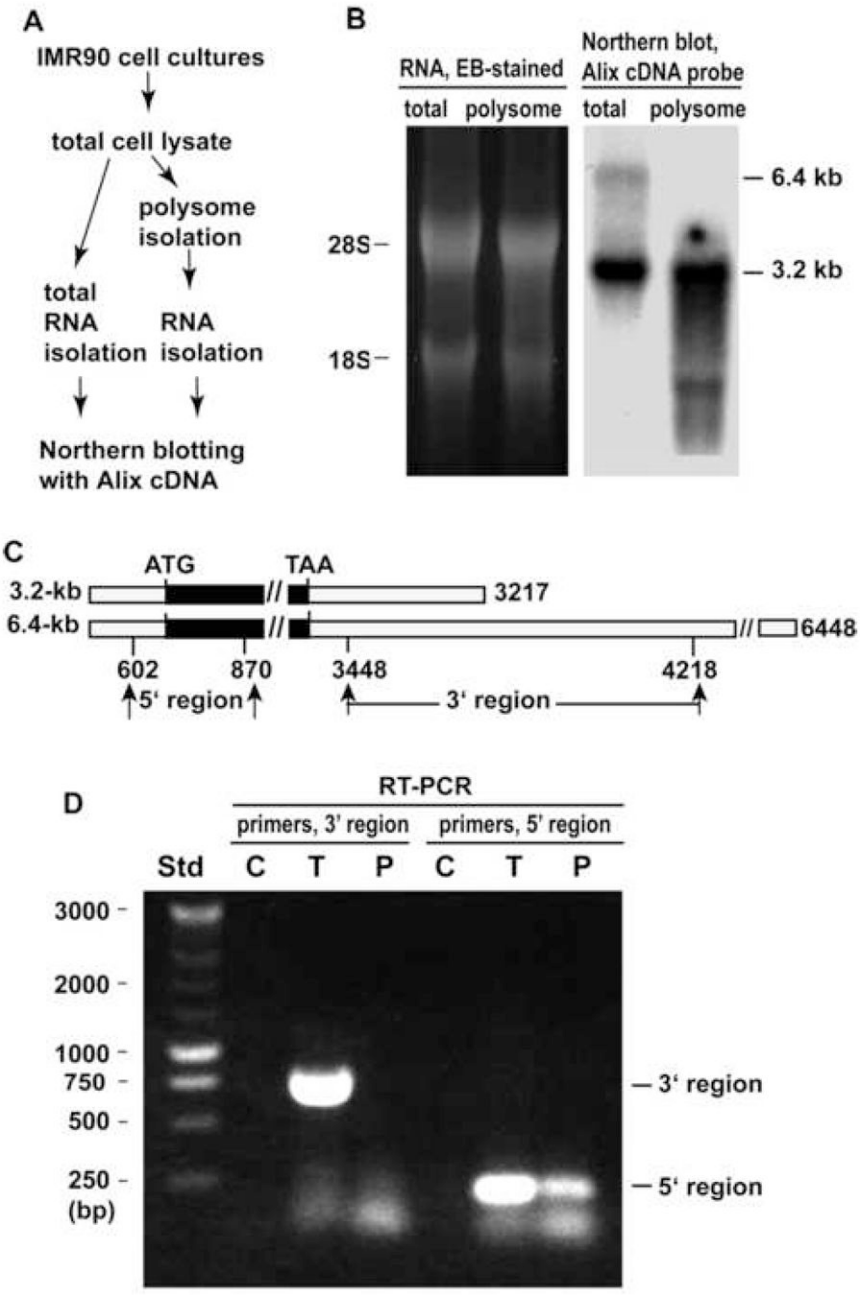


**Figure 6. The 6.4-kb Alix transcript is not engaged in translation**

(A) Diagram for isolation of total and polysomal RNA from IMR90 cells followed by Northern hybridization with Alix cDNA. (B) Left panel: Total and polysomal RNAs were separated by agarose electrophoresis and stained with EtBr. Right panel: Blotted RNAs were hybridized with Alix cDNA. (C) Schematic illustration of the two regions to be amplified by RT-PCR. Filled bars indicate coding regions. (D) Total RNA and polysomal RNA were amplified by RT-PCR with primers for the 5'-region and 3'-region illustrated in (C), and products were separated by agarose gel electrophoresis and stained with EtBr. C: control, in

which only RNA but no reverse transcriptase was provided. T: total RNA was used in reverse transcription. P: polysomal RNA was used in reverse transcription.