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# Molecular Cloning and Characterization of a cDNA for a Novel Phorbol-12-Myristate-13-Acetate-Responsive Gene That Is Highly Expressed in an Adult T-Cell Leukemia Cell Line

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**To identify gene products that might be involved in leukemogenesis of adult T-cell leukemia (ATL), we constructed a cDNA library from an ATL tumor cell line named IKD. By differential plaque hybridization using [$^{32}$P]cDNAs of poly(A)$^+$ RNA from IKD cells and a human T-lymphotropic virus type I-infected T-cell line (C91/PL) as probes and RNA blot analysis, we obtained a single cDNA clone of a gene that is highly expressed in IKD cells. Expression of this gene was also detected in fresh peripheral blood mononuclear cells of several ATL patients but not in those of healthy donors. Sequence analysis showed that the cDNA was that of a previously undescribed gene. On structural analysis of the cDNA (1,897 base pairs), a short open reading frame encoding a polypeptide of 54 amino acid residues was found. Exposure of human peripheral blood mononuclear cells, a T-cell lymphoma cell line (Jurkat), and quiescent human embryonic lung cells to phorbol-12-myristate-13-acetate resulted in rapid, transient expression of 2.0-kilobase mRNA of this gene. This induction of the gene was not inhibited by an inhibitor of protein synthesis, cycloheximide. From these findings, we suggest that this gene, named APR, is a member of the cellular immediate-early-response genes.**

Adult T-cell leukemia (ATL) is etiologically associated with infection by the human T-lymphotropic virus type I (HTLV-I) (18, 42, 59). T cells are known to be immortalized by HTLV-I transmission in vitro (39), and p40$^{tax}$, one of the proteins encoded by the pX region of the HTLV-I genome, is thought to play a key role in their immortalization. Many in vitro studies have shown that this protein is capable of *trans*-activating not only the long terminal repeat of HTLV-I (11, 48, 52) but also several cellular genes of the host cell, such as those of interleukin-2 (IL-2) and IL-2 receptor α chain (IL-2Rα) (9, 38, 51). The immortalization of HTLV-I-infected T cells has been suggested to be caused by an autocrine mechanism of the IL-2/IL-2 receptor system, which is simultaneously induced by the viral transactivator p40$^{tax}$, (9, 22). In fact, the IL-2Rα CD25 (Tac) antigen was found to be expressed on the surfaces of all ATL cells (15). However, the facts that (i) less than 0.1% of HTLV-I carriers develop the disease every year (29, 55, 56) and (ii) the latent period before development of disease is extraordinarily long (57) cannot be explained only by HTLV-I transmission. Malignant cells in an ATL patient were shown to originate from only one HTLV-I-infected T cell (60). Moreover, although a low level of transcripts of the HTLV-I genome was recently detected in fresh peripheral blood mononuclear cells (PBMCs) of ATL patients by the polymerase chain reaction (28), viral antigens have scarcely been detected in freshly isolated ATL cells (10, 17, 20). These observations suggest that immortalization of T cells by HTLV-I infection is a primary event in the pathogenesis of ATL and that some independent subsequent events are required for leukemogenesis. This idea is consistent with the multistep model of carcinogenesis of ATL proposed from epidemiological studies (40). Detection of genetic changes in ATL cells after HTLV-I infection should be helpful in clarifying the molecular mechanism of ATL leukemogenesis. To detect these genetic changes, we constructed a system to compare gene expression in ATL tumor cells with that in HTLV-I-infected T cells. Recently, we established ATL cell lines that showed patterns of rearrangements of T-cell receptor β-chain genes and HTLV-I integration identical to those of the original leukemic cells in vivo (23). One of these established ATL cell lines, named IKD, was used to construct a cDNA library. For detection of genes specifically expressed in the IKD cell line, the cDNA library was screened by differential plaque hybridization with single-stranded cDNAs of poly(A)$^+$ RNA from the IKD and C91/PL cell lines as probes. The latter is a line of HTLV-I-infected T cells established from normal T cells by cocultivation with HTLV-I-producing T cells in vitro (39).

Here, we report the cloning and characterization of a gene that is highly expressed in the IKD cell line and in PBMCs of several ATL patients. We show that this gene was transiently induced by the tumor promoter mitogen phorbol-12-myristate-13-acetate (PMA). The induction of this gene, named APR (ATL-derived PMA-responsive gene), was enhanced by the inhibitor of protein synthesis, cycloheximide, suggesting that it is a novel immediate-early-response gene.

## MATERIALS AND METHODS

**Cell cultures.** Two ATL tumor cell lines, IKD and NKD, were established independently from PBMCs of two ATL patients. These IKD and NKD cells showed patterns of rearrangement of the T-cell receptor β-chain gene and HTLV-I integration identical to that of the original ATL tumor cells (23). These IKD and NKD cells were both maintained in RPMI 1670 containing 10% fetal calf serum (FCS) in the presence of recombinant IL-2 (100 U/ml). The C91/PL cell line was kindly provided by H. Hoshino, Gunma University School of Medicine, Maebashi, Japan. The MT-4 and OCH cell lines were kindly provided by N. Yamamoto, Yamaguchi University School of Medicine, Ube, Japan.

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These cells are HTLV-I-infected T-cell lines that were established by cocultivation of cord blood lymphocytes of normal volunteers with PBMCs of ATL patients. Jurkat cells are a human T-cell lymphoma cell line and were maintained in RPMI 1670 containing 10% FCS. PBMCs were obtained from healthy volunteers and ATL patients and were isolated by Ficoll-Hypaque gradient centrifugation. Nontransformed human embryonic lung (HEL) cells were cultured in Dulbecco modified Eagle medium containing 10% FCS. Before drug treatment or serum activation, HEL cells were cultured at subconfluency in Dulbecco modified Eagle medium containing 0.5% FCS for 48 h to make them quiescent. Phytohemagglutinin (PHA-P; GIBCO Laboratories) and PMA (Sigma Chemical Co.) were used at 10% and 10 ng/ml, respectively. Cycloheximide (Sigma) was used at 10 μg/ml. Drugs were added directly to the culture medium.

**cDNA library preparation and screening.** Total RNA was prepared from IKD and C91/PL cells by the LiCl-urea procedure (2, 26). Poly(A)$^+$ RNA was purified from total RNA after two cycles of selection on an oligo (dT)-cellulose column (3). Double-stranded cDNA was made from 5.0 μg of the poly(A)$^+$ RNA of IKD cells with a cDNA synthesis kit (Bethesda Research Laboratories, Inc.) and then cloned into λgt10 by a standard procedure (21). The unamplified library was plated at low density (~1,000 plaques per 15-cm dish) in 0.7% top agarose and screened by differential plaque hybridization (4). Duplicate sets of nitrocellulose membranes containing recombinant bacteriophage plaques were hybridized to $^{32}$P-labeled cDNA probe ($10^7$ dpm/ml) prepared from poly(A)$^+$ RNA of IKD or C91/PL cells. The membranes were hybridized at 42°C in a solution of 50% (vol/vol) formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M $NaHPO_4$ [pH 7.0], and 0.001 M EDTA), 10% (wt/vol) dextran sulfate, 5× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] Ficoll, 0.02% [wt/vol] polyvinylpyrrolidone, and 0.02% [wt/vol] bovine serum albumin), and heat-denatured salmon sperm DNA (100 μg/ml). After hybridization, the membranes were washed with 1× SSC (0.15 M NaCl and 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) solution at 55°C for 30 min. After tertiary differential screening, plaques chosen for further study were purified. cDNA inserts of the chosen clones were subcloned into the *Eco*RI site of the plasmid vector pTZ18R (United States Biochemical Corp.).

**RNA blot analysis.** Total cellular RNA samples were prepared from several cultured cells or PBMCs by an acid guanidinium thiocyanate-phenol-chloroform procedure (7), fractionated in 1% agarose gel containing 2.2 M formaldehyde, and transferred to a nitrocellulose membrane (12). cDNA inserts subcloned into plasmid pTZ18R were labeled with a multiprime DNA labeling kit (Amersham Corp.) and used as probes. The membranes were hybridized to the probes as described above, washed with 0.2× SSC–0.1% SDS at 65°C for 15 min and autoradiographed.

**Sequence analysis.** cDNA isolates were cloned into pTZ plasmid vectors. Sets of 5′ and 3′ deletions were prepared by using exonuclease III (Takara Shuzo) (16). Overlapping deleted subclones were sequenced by the dideoxynucleotide chain termination method (14, 46) with a Sequenase DNA sequencing kit (United States Biochemical Corp.). Sequences were analyzed with a DNASIS DNA sequence analysis system (Hitachi).

**Primer extension.** The *Pst*I-*Ava*I fragment of the ICP82-23 clone (see Fig. 2A) was labeled at the 5′-terminal end of the *Ava*I site with [γ-$^{32}$P]ATP, using T4 polynucleotide kinase (Takara Shuzo) and used as a primer. The primer was hybridized to 30 μg of poly(A)$^+$ RNA from Jurkat cells stimulated with PMA (10 ng/ml) for 2 h. Hybridization and subsequent reactions were carried out as described previously (37). Primer extension products were analyzed by electrophoresis on an 8% polyacrylamide sequencing gel.

**In vitro transcription and translation.** The 1.9-kilobase (kb) *Eco*RI cDNA insert of clone ICP82-23 was subcloned into the *Eco*RI site of the pTZ18R vector, containing the T7 phage promoter just upstream of the multicloning region. The recombinant plasmid that expressed the sense-strand RNA of the full-length cDNA insert under the control of the T7 phage promoter was designated pICP82-23FL. Four deletion mutants of the cDNA (shown in Fig. 3A) were constructed as follows. pICP82-23dX was constructed from pICP82-23FL by deleting the 300-base-pair (bp) *Xba*I fragment in the 3′-terminal portion of the insert. pICP82-23ED was obtained by subcloning the 550-bp *Eco*RI-*Dra*I fragment of the 5′-terminal portion of the cDNA into the *Eco*RI-*Sma*I site of the pTZ18R vector. pICP82-23VX and pICP82-23AV were constructed from pICP82-23FL by deleting the 1,500-bp *Eco*RV-*Xba*I fragment and the 250-bp *Acc*III-*Eco*RV fragment, respectively, and ligation after filling in the cohesive ends of the restricted sites by the Klenow fragment. All purified plasmids were linearized by cutting the unique *Hin*dIII sites of multicloning regions downstream of the insert and then used as templates in *in vitro* transcription. RNA transcripts were synthesized in vitro with T7 RNA polymerase (United States Biochemical Corp.) under the conditions specified by the supplier with addition of m$^7$GpppG (Pharmacia) to the reaction mixture. The transcripts were translated in wheat germ extracts (Amersham) containing 100 mM potassium acetate in the presence of [$^{35}$S]methionine. The translation products were analyzed by tricine-SDS-polyacrylamide gel electrophoresis (47), with acrylamide cross-linker (FMC Biochemicals) instead of bisacrylaide, and fluorography.

**Nucleotide sequence accession member.** The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank under accession number D90070.

## RESULTS

**Preparation and screening of a cDNA library from IKD cells.** A λgt10 cDNA library was prepared with poly(A)$^+$ RNA from IKD cells. A library of approximately $8.7 \times 10^6$ independent phages was obtained. For detection of cDNAs that were expressed or depressed specifically in IKD cells, phages of the library were plated at low density without amplification and screened by differential plaque hybridization in which duplicate lifts were probed with $^{32}$P-labeled single-stranded cDNA prepared from poly(A)$^+$ RNA from IKD and C91/PL cells, respectively. Plaques giving hybridization signals of different intensity with the two probes were selected for a second screening. The hybridization patterns of the two probes in the first screening were quite similar. Of about 40,000 plaques screened, four plaques that hybridized more strongly with the IKD cell probe than with the C91/PL cell probe and three plaques that hybridized more strongly with the C91/PL cell probe were obtained after tertiary screening. For confirmation that the selected cDNA clones were those of genes that were regulated specifically in IKD cells, RNA blot analysis was performed, using the cDNA inserts as probes. Total cellular RNA extracted from IKD, NKD, and C91/PL cells and from three other HTLV-I-infected T-cell lines (MT-2, MT-4, and OCH) were hybrid-

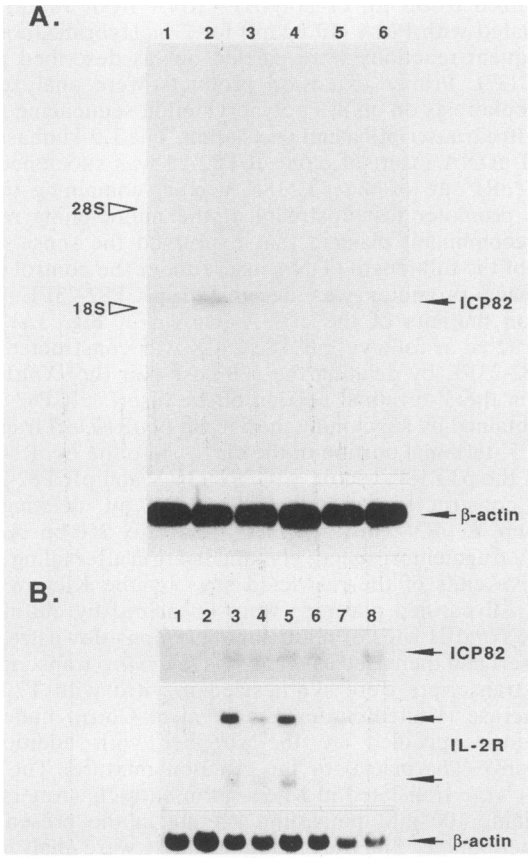


FIG. 1. ICP82 mRNA levels in T-cell lines and human PBMCs. (A) RNA blot analysis of ATL tumor cell lines and HTLV-I-infected T-cell lines. Lanes: 1, NKD cells; 2, IKD cells; 3, C91/PL cells; 4, MT-2 cells; 5, MT-4 cells; 6, OCH cells. Hybridization was performed sequentially with a ICP82 cDNA probe (*Eco*RI fragment of ICP82-1 cDNA insert) and a β-actin cDNA probe. The positions of 18S and 28S rRNAs are indicated. (B) RNA blot analysis of PBMCs from healthy volunteers and ATL patients. Lanes: 1 and 2, PBMCs from healthy volunteers; 3 through 8, PBMCs from ATL patients. Hybridization was performed sequentially to a ICP82 cDNA probe, a IL-2Rα cDNA probe, and a β-actin cDNA probe. A 10-μg sample of total RNA was electrophoresed in 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane.

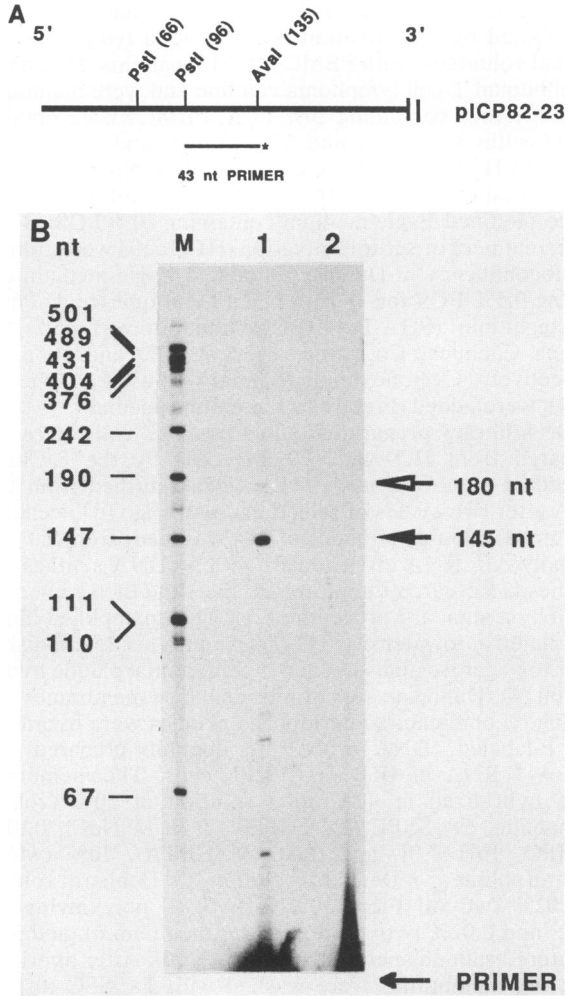


FIG. 2. Primer extension analysis. Reactions were performed with and without 30 μg of poly(A)$^+$ RNA from Jurkat cells treated with PMA (10 ng/ml) for 2 h (lanes 1 and 2, respectively). The *Pst*I-*Ava*I fragment of pICP82-23 was labeled with [γ-$^{32}$P]ATP at its 5′ terminus (marked with a star in panel A) and used as a primer. End-labeled *Msp*I digests of pUC119 were used as size markers (lane M). nt, Nucleotides.

ized with the probes. IKD cells and NKD cells were established independently from PBMCs of different ATL patients (23). Four of seven cDNA clones (one expressed more and three expressed less in IKD cells than in C91/PL cells) represented sequences whose expression was regulated specifically in IKD cells, whereas the remainder were expressed at the same level in some HTLV-I-infected T cells as in IKD cells. We could not find a clone that was expressed in both IKD cells and NKD cells. For investigation of the expression patterns of these four clones in ATL cells in vivo, total RNA from PBMCs of ATL patients were analyzed by RNA blot analysis, using the cDNA inserts as probes. We found that one clone (termed ICP82-1), which was highly expressed in IKD cells (Fig. 1A), was expressed in PBMCs from 6 of 13 ATL patients but not at a detectable level in any PBMCs from 10 healthy donors examined (Fig. 1B). The mRNAs corresponding to the cDNAs of the other three clones were found to be expressed at similar levels in all PBMC samples (data not shown). The ICP82-1 clone was finally selected for further analysis.

**Characterization of ICP82 cDNA clones and corresponding mRNA.** The ICP82-1 clone contained an insert of approximately 1.3-kb cDNA which hybridized with mRNA of about 2.0 kb (Fig. 1). Therefore, to obtain full-length cDNA clones, we rescreened the same cDNA library by using the $^{32}$P-labeled cDNA insert of clone ICP82-1 as a probe. Among $10^5$ plaques, we obtained 24 positive clones (designated ICP82-2 through ICP82-25). ICP82-7 and ICP82-23, which contained the longest cDNA inserts (about 1.9 kb), were further characterized in addition to ICP82-1. Sequence analysis of ICP82 cDNA is described later. Although the cDNA insert of clone ICP82-23 spans 1,885 bp [excluding the poly(A) tail] and is the longest of the three cDNA inserts, we could not find a long open reading frame in its cDNA sequence. Therefore, to verify that the cDNA sequence obtained covers the full length of the mRNA, we performed primer extension analysis. Figure 2B shows the result of primer

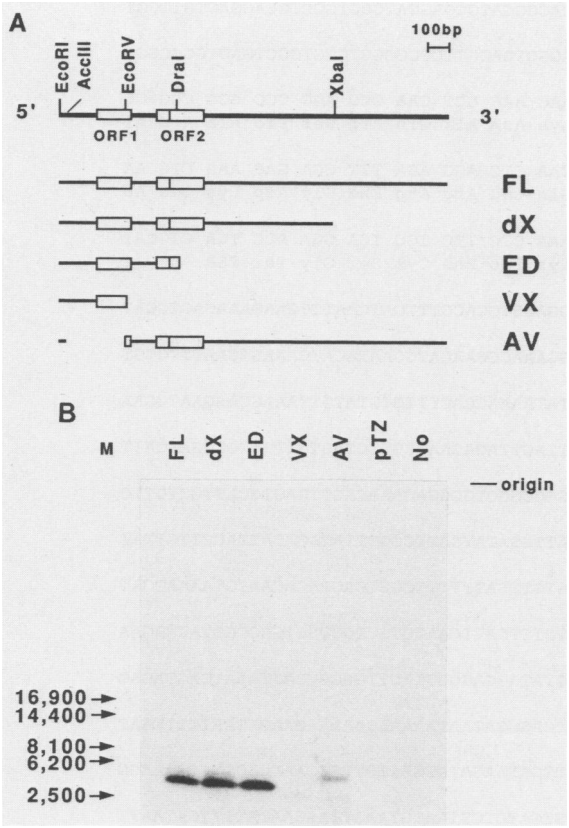


FIG. 3. In vitro translation of ICP82 transcripts. (A) Plasmid constructions and restriction sites used in the construction. The presumptive open reading frames (ORF1 and ORF2) are indicated by boxes. RNAs transcribed in vitro from pICP82-23FL, pICP82-23dX, pICP82-23ED, pICP82-23VX, and pICP82-23AV (FL, dX, ED, VX, and AV, respectively) were translated in wheat germ extracts in the presence of [$^{35}$S]methionine, and the products were analyzed on tricine–SDS–16% acrylamide gel (46). (B) Lanes: FL, dX, ED, VX, and AV, translation products of transcripts from FL, dX, ED, VX, and AV, respectively; pTZ, translation products obtained when transcripts from pTZ18R vector linearized by *Hin*dIII digestion were used in the in vitro translation system; No, translation products obtained in the wheat germ extract without the transcript; M, molecular weight markers.

extension analysis using the *Pst*I-*Ava*I fragment of the cDNA insert of ICP82-23 as a primer. Reverse transcription of poly(A)$^+$ RNA from Jurkat cells indicated that the 5′ end of the ICP82 mRNA is about 145 and 180 nucleotides upstream of the primer sequence (Fig. 2B, lane 1). Therefore, the predominant 5′ end of the mRNA is about 10 nucleotides upstream of the 5′ end of the ICP82-23 cDNA sequence. A single minor product of 180 nucleotides is probably derived from a minor mRNA species. From these results, we concluded that the cDNA insert of clone ICP82-23 covers nearly the full length of the ICP82 mRNA.

**Synthesis of the ICP82 cDNA protein product in vitro.** We could not find a long open reading frame in the ICP82 cDNA sequence, but there were two short reading frames located at positions 174 to 335 (ORF1) and 478 to 696 (ORF2) (Fig. 3 and 4). A second ATG codon (position 535) was also found in the frame of ORF2. Although the ATG codon at position 174 is the first available ATG codon in the cDNA sequence and stop codons were found in all three frames upstream of the ATG codon, the nucleotide sequences around the ATG codon did not conform to the consensus sequence of the eucaryotic translation initiation signal (30). To confirm the open reading frame of the ICP82 cDNA sequence, we carried out in vitro transcription and translation experiments using various deletion mutants of the ICP82-23 cDNA insert as templates. As shown in Fig. 3, a single major protein with an $M_r$ of about 4,000 was generated from the pICP82-23FL-, pICP82-23dX-, and pICP82-23ED-derived transcripts (Fig. 3B). The translation product of the pICP82-23VX-derived transcript of $M_r$ 3,200 was smaller than those of the aforementioned three transcripts, consistent with the fact that ORF1 in the pICP82-23VX construct is truncated, lacking the carboxyl-terminal nine amino acid residues and immediately followed by a stop codon in frame (Fig. 3B). The major product of nearly full length ICP82 transcript (pICP82-23FL-derived product; $M_r$ 4,000) was not detected with pICP82-23AV, in which ORF1 was almost completely deleted (Fig. 3B), although a minor product ($M_r$ 4,500) was detected, possibly representing the translation product derived from ORF2. From these results, it was concluded that the translation product of $M_r$ 4,000 was derived from ORF1. The molecular weight of the ORF1-encoding polypeptide is calculated from the deduced amino acid sequence (Fig. 4) to be 6,012, but the discrepancy in molecular weight could be due to abnormal mobility of the polypeptide in this gel electrophoresis system. Therefore, we concluded that ORF1 is the major protein coding region of the ICP82 cDNA sequence.

**Sequence analysis of the ICP82 cDNA clone.** Figure 4 shows the composite nucleotide sequence of the ICP82 cDNA clone together with the deduced amino acid sequence. The cDNA sequence is composed of 173 bp of a 5′ noncoding region, 162 bp of a coding region (ORF1), and 1,550 bp of a 3′ noncoding region. The ICP82 product contains 54 amino acids with a calculated molecular weight of 6,012. Comparison of the cDNA sequence and the deduced amino acid sequence by DNA and protein homology search programs did not reveal any homology with published sequences in GenBank (R58.0, December 1988), EMBL (R17.0, November 1988), or PIR (R18.0, September 1988; National Biomedical Research Foundation) data bases, indicating that the ICP82 clone represent a newly discovered gene. The ICP82 product contains 11 basic residues (6 lysines and 5 arginines) and 4 acidic residues (3 glutamic acids and 1 aspartic acid) and therefore has a net positive charge. The four acidic residues are clustered in the central region of the polypeptide. Examination of a Hopp-Woods hydrophilicity plot (19) of the deduced amino acid sequence indicated a hydrophilic nature of the polypeptide as a whole (data not shown), with no long stretch of hydrophobic residues in any part of the primary structure. In the 3′ noncoding region, a putative polyadenylylation signal, AGTAAA (positions 1859 to 1864) (5), is present 21 bases upstream of the poly(A) tail (Fig. 4, underline). The 3′ noncoding region contains four repeats of an ATTTA pentamer motif (positions 1666 to 1670, 1743 to 1747, 1801 to 1805, and 1819 to 1823) and a TTATTTAT octamer motif (positions 1551 to 1558) (Fig. 4, double underlines), which have been identified as common sequences in the cDNAs for a number of cytokines, lymphokines, and proto-oncogenes (6, 49).

**Regulation of ICP82 mRNA expression.** Basal expression of ICP82 mRNA was relatively low in all cultured cell lines examined (Fig. 1A and other data not shown). Furthermore, ICP82 mRNA was not detected in any PBMCs from healthy volunteers (Fig. 1B). To test the possibility of the induction of ICP82 gene expression by mitogens, we treated several cell lines with mitogens and then examined them by RNA

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   1  CGGGCACTCACCGTGTGTAGTTGGCATCTCCGCGCGTCCGGACACCCGATCCCAGCATCCCTGCCTGCAGGACTGTTCGT

  81  GTTCAGCTCGCGTCCTGCAGCTGTCCGAGGTGCTCCAGTTGGAGGCTGAGGTTCCCGGGCTCTGTCGCTGAGTGGGCGGC

 161  GGCACCGGCGGAG ATG CCT GGG AAG AAG GCG CGC AAG AAC GCT CAA CCG AGC CCC GCG CGG GC
   1               Met Pro Gly Lys Lys Ala Arg Lys Asn Ala Gln Pro Ser Pro Ala Arg Al

 224  T CCA GCA GAG CTG GAA GTC GAG TGT GCT ACT CAA CTC AGG AGA TTT GGA GAC AAA CTG AA
  17  a Pro Ala Glu Leu Glu Val Glu Cys Ala Thr Gln Leu Arg Arg Phe Gly Asp Lys Leu As

 284  C TTC CGG CAG AAA CTT CTG AAT CTG ATA TCC AAA CTC TTC TGC TCA GGA ACC TGA CTGCAT
  37  n Phe Arg Gln Lys Leu Leu Asn Leu Ile Ser Lys Leu Phe Cys Ser Gly Thr TER

 345  CAAAAACTTGCATGAGGGGACTCCTTCAAAAGAGTTTTCTCAGGAGGTGCACGTTTCATCAATTTGAAGAAAGACTGCAT

 425  TGTAATTGAGAGGAATGTGAAGGTGCATTCATGGGTGCCCTTGGAAACGGAAGATGGAATACATCAAAGTGAATTTCTGT

 505  TCAAGTTTTCCCAGATTATCATTCTTTGGGATGAGAGAACATTATAAAACCACTTTGTTTATTTTAAAGCAAGAATGGAA

 585  GACCCTTGAAAATAAAGAAGTAATTATTGACACATTTCTTTTTTACTTAGAGAATCGTTCTAGTGTTTTTGCCGAAGATT

 665  ACCGCTGGCCTACTGTGAAGGTAGATGACCTGTGATTAGACTGGGCGGCTGGGGAGAAACAGTTCAGTGCATTGTTGTTG

 745  TTGCTGTTTTTGGTGTTTTGCTTTTCAGTGCCAACTCAGCACATTGTATATGATTCGGTTTATACATATTACCTTGTTAT

 825  AATGAAAAAACTCATTCTGAGAACACTGAAATGTTATACTCAGTGTTGATTTCTTCGGTCACTACACAACGTAAAATCAT

 905  TTGTTTCTTTTGACTCAAATTGTATTGCTTCTGTTCAGATGATCTTTCATTCAATGTGTTCCTGTTGGGCGTTACTAGAA

 985  ACTATGGAAAACTGGAAAATAACTTTGAAAAAATTGGATAAAGTATAGGAGGGTTACTTGGGGCCAGTAAATCAGTAGAC

1065  TGAACATTCAATATAATAAAAGAACATGGGGATTTTGTATAACCAGGGATAATAAAAAGAAAAAGAAGTTAATTTTTAAT

1145  TGATGTTTTTGAAACTTAGTAGAACAAATATTCAGAAGTAACTTGATAAGATATGAATGTTTCTAAAGAGTTTCTAAAGG

1225  TTCGAAATGCTCCTTGTCACATTAGTGTGCATCCTACAAAAAGTGATCTCTTAATGTAAATTAAGAATATTTTCATAATT

1305  GGAATATACTTTTCTTAAAAAAAAGGAACAGTTAGTTCTCATCTAGAATGAAAGTTCCATATATGCATTGGTGAATATAT

1385  ATGTATACACATACTTACATACTTATATGGGTATCTGTATAGATAATTTGTATTAGAGTATTATATAGCTTCTTAGTAGG

1465  GTCTCAAGTAAGTTCATTTTTTTTATCTGGGCTATATACAGTCCTCAAATAAATAATGTCTTGATTTTATTTCAGCAGGA

1545  ATAATTTTATTTATTTTGCCTATTTATAATTAAAGTATTTTTCTTTAGTTTGAAATGTGTATTAAAGTTACATTTTTGAG

1625  TTACAAGAGTCTTATAACTACTTGAATTTTTAGTTAAAATGTCTTAATGTAGGTTGTAGTCACTTTAGATGGAAAATTAC

1705  CTCACATCTGTTTTCTTCAGTATTACTTAAGATTGTTTATTTAGTGGTAGAGAGATTTTTTTTTTCAGCCTAGAGGCAGC

1785  TATTTTACCATCTGGTATTTATGGTCTAATTTGTATTTAAACATATGCACACATATAAAAGTTGATACTGTGGCAGTAAA

1865  CTATTAAAAGTTTTCACTGTT
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FIG. 4. Nucleotide sequence and deduced amino acid sequence of the ICP82 cDNA clone. Numbers on the left are those of the first nucleotide listed on that line. Amino acids are numbered from the first methionine. The translation termination codon is indicated by TER. The presumptive polyadenylation signal (AGTAAA) is underlined. The sequence motifs commonly found in 3′ noncoding regions of cytokine, lymphokine, and protooncogene mRNAs (6, 48) are indicated by double underlines.

blot hybridization. ICP82 mRNA was rapidly induced by phytohemagglutinin (PHA) plus PMA in PBMCs from a healthy volunteer (Fig. 5A). The mRNA was detected within 2 h after treatment, although it was not found in untreated PBMCs (Fig. 5A). The level of induced mRNA gradually decreased, returning to the initial level by 24 h after treatment (Fig. 5A). Next, we tested the mitogenic induction of expression of this gene in the Jurkat cell line, in which several genes, including the IL-2 and IL-2 receptor genes, are known to be inducible (13). Expression of this gene in Jurkat cells was elevated within 1 h and reached a peak about 2 h after treatment with PHA plus PMA (Fig. 5B), as in PBMCs. About a 15-fold increase over the steady-state level was observed when the cells were stimulated by these mitogens for 2 h (Fig. 5B). This gene responded mainly to PMA alone, although it responded slightly to PHA alone (Fig. 5B). Thus, we named this gene APR (ATL-derived PMA-responsive gene). The same kinetic pattern of induction of APR mRNA by PMA treatment was seen in nontransformed HEL fibroblasts (Fig. 5C). Expression of the APR gene was not detected in serum-deprived HEL cells, as was found for resting PBMCs. The inducibility of this gene was enhanced in the presence of an inhibitor of protein synthesis, cycloheximide (Fig. 5C). Accumulation of the mRNA was also found in cells treated with cycloheximide alone (Fig. 5C). These data indicate that expression of the APR gene is transiently and rapidly induced by PMA treatment without de novo protein synthesis. The same pattern of expression of

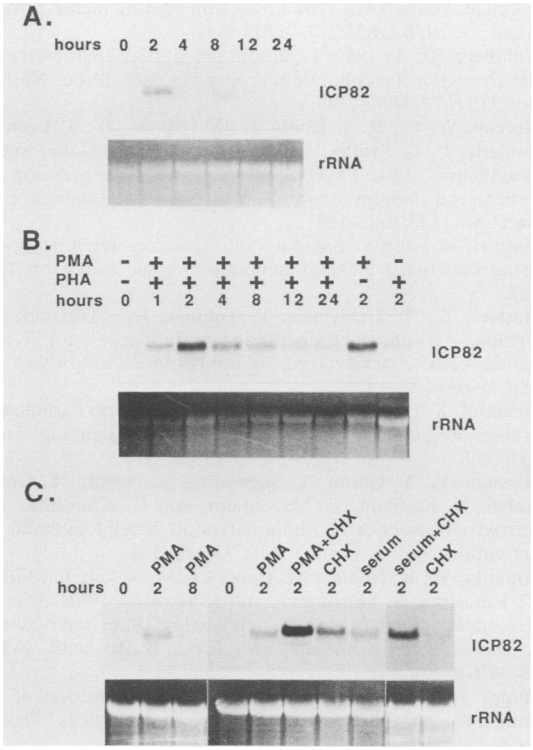


FIG. 5. Kinetics of induction of ICP82 mRNA in PBMCs from a healthy volunteer (A), Jurkat cells (B), and serum-starved HEL cells (C). (A) PBMCs were incubated with PMA (10 ng/ml) plus PHA (1%) for various times. (B) Jurkat cells were treated with PMA (10 ng/ml), PHA (1%), or both for various times. (C) Serum-starved HEL cells were induced with PMA (10 ng/ml) for 0, 2, and 8 h and were treated with PMA (10 ng/ml) or 20% FCS in the presence or absence of cycloheximide (10 μg/ml) for 2 h. Total RNA (10 mg/ml) was analyzed by RNA blot hybridization as for Fig. 1, using the *Eco*RI fragment of the ICP82-23 cDNA insert as a probe, and equalized for 28S rRNA.

the APR gene was also seen in HEL cells after activation with serum instead of PMA (Fig. 5C).

## DISCUSSION

We have identified and characterized a cDNA for a gene that was highly expressed in an ATL-derived tumor T-cell line (IKD cells) and have named it APR (ATL-derived PMA-responsive gene). We found that the APR cDNA sequence contained four ATTTA pentamer motifs and a TTATTTAT octamer motif in the 3′ noncoding region (Fig. 4). Current evidence suggests that these motifs function in posttranscriptional regulation of transient gene expression by conferring instability to the mRNAs (41, 49, 58). Although we do not yet know whether these motifs are sufficient to destabilize the APR mRNA, we think that expression of the APR gene may share a common mode of regulation with other genes with these motifs. As expected, rapid, transient induction of APR mRNA expression in response to a mitogenic stimulus (PMA) was observed in human PBMCs, a Jurkat cell line, and HEL cells. Moreover, serum had the same effect as PMA on serum-starved HEL cells. The induction was enhanced rather than inhibited by an inhibitor of protein synthesis, cycloheximide. A low level of expression of APR mRNA was detected in all proliferating human cells tested, but expression of APR mRNA was almost completely suppressed in primary human resting PBMCs and serum-starved HEL cells. Human resting PBMCs represent normal cells in a physiological quiescent state ($G_0$) (43, 53), and serum-starved HEL cells are also thought to be mostly quiescent, judging from their level of [$^3$H]thymidine incorporation (data not shown). The pattern of induction of APR gene expression upon addition of PMA or serum to quiescent cells was similar to that of the c-*fos* (42) or c-*myc* (27, 43) gene, although the maximum level of induction of the APR gene was roughly estimated to be clearly much lower than that of c-*fos*. These data indicate that the APR gene is a member of the cellular immediate-early-response genes (33) and should be expressed during the $G_0/G_1$ transition (cell cycle reentry).

Several cDNA clones for cellular immediate-early response genes, including c-*fos* and c-*myc* genes, have been isolated (1, 32, 33, 61). The immediate-early response genes that have been well characterized can be divided into two main classes. One class includes genes for presumptive nuclear factors, such as c-Fos (43), c-Myc (27, 43), c-Jun (45), and a zinc finger-containing protein (8, 31, 54), and the other class includes genes for presumptive secretory factors, such as Act-2 (35). The predicted APR protein (54 amino acid residues) is thought to be hydrophilic, and positively charged, as a whole (Fig. 4). It does not contain a long hydrophobic region that could possibly act as a signal peptide of a secretory protein. On the other hand, the amino-terminal portion of this hydrophilic polypeptide is rich in basic residues and proline residues (Fig. 4). This characteristic feature, especially from residue 2 to residue 8 (-Pro-Gly-Lys-Lys-Ala-Arg-Lys-), resembles that of nuclear localization signals in simian virus 40 large T antigen (-Pro-Lys-Lys-Lys-Arg-Lys-Val-) (24, 25), polyomavirus large T antigen (-Val-Ser-Arg-Lys-Arg-Pro-Arg-) (44), and adenovirus type 5 E1A proteins (-Ser-Cys-Lys-Arg-Pro-Arg-COOH) (36). All of these sequences are highly positively charged and contain a proline residue, but little direct homology among these sequences was apparent. This suggests that the APR protein may be a nuclear factor. Biochemical and immunocytochemical studies are now under way to detect the APR product in vivo and to examine its subcellular localization.

The high expression of APR mRNA in IKD cells is unlikely to be caused by HTLV-I infection, because both the IKD and the C91/PL cells used in this study were infected by the virus. The low level of expression of the APR gene in NKD cells, which were established from a different ATL patient from IKD cells, suggests that the aberrant accumulation of the APR mRNA is a specific feature of IKD cells (Fig. 1A). No drastic rearrangement, deletion, or amplification of the APR gene or integration of the HTLV-I genome close to the APR gene was detected in IKD cells by genomic Southern blot analysis using several restriction endonucleases (data not shown). Therefore, some change in regulation of gene expression may have occurred in IKD cells. APR mRNA was not detected in PBMCs from healthy volunteers, but expression of the APR gene was observed in the PBMCs from 6 of 13 ATL patients (Fig. 1B and other data not shown). The finding that APR gene expression was detected in PBMCs from some but not all ATL patients seems analogous with the difference in the level of expression of the APR mRNA in IKD and NKD cells and suggests that leukemic cells in some ATL patients may express a high level of APR mRNA. As the APR gene may be expressed in a cell cycle-dependent manner, as mentioned above, an alternative interpretation for the difference in the levels of

APR mRNA in PBMCs from different ATL patients may be that the numbers of proliferating PBMCs in these ATL patients differ. In fact, wide variation in the proportion of activated PBMCs in ATL patients has been demonstrated at different clinical stages by using a series of monoclonal antibodies to the cell surface membrane and cell nucleus (50). Unlike APR mRNA, IL-2Rα mRNA was detected in the PBMCs of all ATL patients, consistent with the results of a previous immunofluorescence study (15) (Fig. 1B). However, the expression of IL-2Rα mRNA, like that of APR mRNA, could be induced in T cells by PMA alone (34). This difference indicates that APR gene expression in the PBMCs from several ATL patients is regulated by a different mechanism from IL-2Rα gene expression.

It will be interesting to determine whether there is any correlation between the synthesis of excess APR mRNA and neoplasmic transformation; in this connection, analysis of the function of the APR gene product is also necessary.

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