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Molecular cloning and nucleotide sequence of a full-length cDNA for human α enolase

(cDNA expression library/antibody screening/glycolytic enzyme/mitogenic stimulation/c-myc protein)

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ABSTRACT **We previously purified a 48-kDa protein (p48) that specifically reacts with an antiserum directed against the 12 carboxyl-terminal amino acids of the c-*myc* gene product. Using an antiserum directed against the purified p48, we have cloned a cDNA from a human expression library. This cDNA hybrid-selects an mRNA that translates to a 48-kDa protein that specifically reacts with anti-p48 serum. We have isolated a full-length cDNA that encodes p48 and spans 1755 bases. The coding region is 1299 bases long; 94 bases are 5′ noncoding and 359 bases are 3′ noncoding. The cDNA encodes a 433 amino acid protein that is 67% homologous to yeast enolase and 94% homologous to the rat non-neuronal enolase. The purified protein has been shown to have enolase activity and has been identified to be of the α type by isoenzyme analysis. The transcriptional regulation of enolase expression in response to mitogenic stimulation of peripheral blood lymphocytes and in response to heat shock is also discussed.**

We previously reported (1) the purification of a 48-kDa protein that crossreacts with an antiserum against a synthetic peptide corresponding to the carboxyl terminus of the human cellular myc protein (1). This protein was characterized as having a basic isoelectric point and cytoplasmic localization. The protein is present in a relatively high amount in all the cell types analyzed with the exception of normal resting lymphocytes, where it is detectable only after mitogenic stimulation (1). We now have used an antiserum prepared against purified p48 to screen a human cDNA expression library, and we have isolated a full-length cDNA coding for p48. The predicted amino acid sequence of p48 is 94% homologous to the amino acid sequence of rat non-neuronal enolase (2), identifying p48 as a subunit of human enolase.

Enolase is a glycolytic enzyme (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11), and three classes of isoenzymes have been identified in mammalian tissues. Each isoenzyme is a homodimeric protein composed of two α, β, or γ subunits (3). Isoenzyme α is present in most tissues, β is localized in muscle tissue, and γ is found only in nervous tissue (3). We have demonstrated enolase activity in the purified p48 and have identified the protein as the α isoenzyme, which is the prevalent isotype in human lymphoid cell lines.

MATERIALS AND METHODS

Screening of the Expression Library. The construction of a cDNA expression library from the human T-cell line Jurkat has been reported (4). The library was screened using the anti-p48 serum (1) according to the procedure of Helfman *et al.* (5), with the following modifications. Approximately 10^3 bacterial colonies per filter were lysed with $CHCl_3$ vapor and then incubated overnight in 10 ml of 50 mM Tris/HCl, pH 7.5/150 mM NaCl/5 mM $MgCl_2$ containing 1% bovine serum albumin (BSA), 0.25% gelatin, and 1 μg of DNase and 40 μg of lysozyme per ml. The filters were rinsed three times in buffer A (50 mM Tris, pH 7.6/150 mM NaCl/5 mM EDTA/0.25% gelatin) and incubated for 1 hr at room temperature with 10 ml of the rabbit antiserum diluted in buffer A supplemented with 1% BSA. The filters were washed with buffer A containing 0.05% Nonidet P-40 at room temperature (three changes, 10 min per wash). ^{125}I-labeled donkey F(ab′)$_2$ fragments specific for rabbit IgG (Amersham) were used as second antibody (1–5 × 10^6 cpm per filter). After 1 hr of incubation at room temperature, the filters were washed as described above and subjected to autoradiography. The library was rescreened, as described by Grunstein and Hogness (6), using a ^{32}P-labeled cDNA clone (pSA082) isolated by the anti-p48 screening, in order to isolate a full-length cDNA clone.

Analysis of Protein Expressed in Bacteria. Overnight cultures were diluted 1:20 in Luria–Bertani (LB) broth and grown for 2 hr at 37°C. Total cell extracts were prepared by lysing the bacteria in Laemmli sample buffer containing 6 M urea, according to Courtney *et al.* (7). Aliquots were analyzed by immunoblotting with anti-p48 serum as described (1).

Hybrid-Selected Translation Assay. Poly(A)$^+$ RNA from the Burkitt lymphoma cell line Daudi was hybrid-selected according to the procedure of Parnes *et al.* (8) and translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories). Translation products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis before and after immunoprecipitation with anti-p48 antibodies (9).

Nucleotide Sequencing. Restriction fragments were subcloned into M13 mp18 or mp19 and sequenced by the dideoxy chain-termination procedure of Sanger *et al.* (10). Large fragments were directionally sequenced after progressive digestion with exonuclease III (New England Biolabs) as described by Henikoff (11).

Protein Purification and Enzymatic Assay. p48 (enolase) was purified from cell lysates as previously described (1), with the following modifications in order to preserve enzymatic activity. Enolase-enriched fractions from preparative isoelectric focusing (1) were further purified through a Sephadex G-150 column (1 × 230 cm) in 10 mM Tris, pH 7.6/4 mM $MgSO_4$/0.1 mM EDTA. Enolase activity was measured in the collected fractions according to Baranowski and Wolna (12) and expressed as arbitrary units/ml. Isoenzyme analysis was carried out as described by Chen and Giblett (13), with minor modifications.

RNA Blot Analysis. Cellular cytoplasmic RNA, isolated according to Berger and Birkenmeier (14), was denatured in 1 M glyoxal at 50°C for 1 hr and electrophoresed in a neutral 1% agarose gel. The fractionated RNAs were then electro-

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Abbreviations: kb, kilobase(s); bp, base pair(s).
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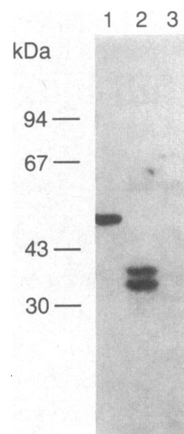

FIG. 1. Immunological detection of p48-related peptides encoded by cDNA clone pSA082. *E. coli* strain JM109 carrying pSA082 or pUC8 vector was lysed as described in *Materials and Methods*. Proteins were electrophoresed in a $NaDodSO_4$/10% acrylamide gel, transferred to nitrocellulose, and allowed to react with anti-p48 antibodies (1). For comparison, lysate from the Burkitt lymphoma cell line Daudi was also analyzed (lane 1). Lane 2: JM109 carrying pSA082. Lane 3, JM109 carrying pUC8. Molecular size markers were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

blotted onto Nytran membranes (Schleicher & Schuell) and hybridized with ^{32}P-labeled cDNA (pSA082) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Isolation of cDNA Clones for Human p48 (Enolase). The human T-cell cDNA expression library in *Escherichia coli* JM109 was first screened with an antiserum against purified p48. A single strongly positive clone, pSA082, with an insert size of 1.5 kilobases (kb) was obtained by screening approximately 20,000 bacterial colonies. In order to identify the p48-related protein encoded by pSA082, we analyzed the translation products present in bacteria carrying this plasmid. Fig. 1 shows the reaction of the anti-p48 antibodies with the peptide expressed by pSA082, after electrophoresis and electroblotting onto nitrocellulose filter. As shown in lane 2, anti-p48 detected two immunologically related polypeptides with apparent molecular masses of 34 and 38 kDa. The intensity of the reaction of the two peptides with the antiserum was comparable to that observed with p48 in Daudi cell lysates (lane 1), and no crossreacting proteins were detected in the control lysate of bacteria containing pUC8 plasmid lacking an insert (lane 3). The identification of peptides

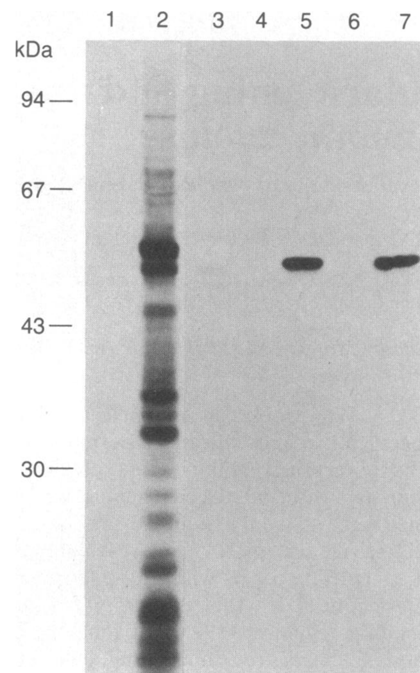

FIG. 2. Identification of human p48 cDNA by hybrid-selected translation. Poly(A)$^+$ RNA from Daudi cells was hybridized to pSA082 or pBR322 that had been bound to nitrocellulose filters. The hybridized RNA was eluted and translated in a reticulocyte lysate system containing [^{35}S]methionine. The products were either immunoprecipitated or directly analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by autoradiography. Lanes: 1, *in vitro* translation without added poly(A)$^+$; 2, translation products of 0.3 μg of poly(A)$^+$; 3, products of RNA hybrid-selected with pBR322; 4, immunoprecipitation with anti-p48 of products hybrid-selected with pBR322; 5, products of RNA hybrid-selected with pSA082; 6, immunoprecipitation with preimmune serum of products hybrid-selected with pSA082; 7, immunoprecipitation with anti-p48 antibodies of products hybrid-selected with pSA082.

smaller than 48 kDa suggested that pSA082 does not contain the complete cDNA encoding p48. The expression of two polypeptides from the same cDNA clone is due to the presence of additional strong internal AUG initiation signals, and in fact both of these polypeptides initiate from internal AUG codons and not from the one encoded in the pUC8 vector (unpublished data). To substantiate that pSA082 encoded part of the mRNA for p48, we used the hybrid-selected translation assay (Fig. 2). The translation product from mRNA specifically selected with pSA082 plasmid DNA was a 48-kDa polypeptide (Fig. 2, lane 5). This protein was

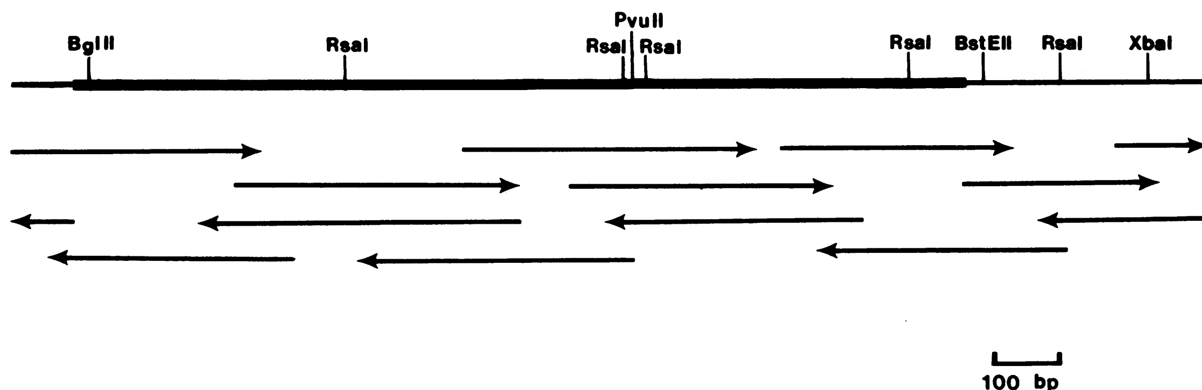

FIG. 3. Partial restriction map and sequence-determination strategy for pH48. The protein-encoding region is indicated by the thick line. Arrows indicate direction and extent of nucleotide sequence determination for each fragment analyzed. bp, Base pairs.

specifically immunoprecipitated by anti-p48 antibodies (lane 7) and not by preimmune serum (lane 6). A faint band at about the same molecular mass was observed in the control sample (lane 3), which was hybrid-selected against pBR322; however, this protein was not immunoprecipitated with anti-p48 serum (lane 4) but is an endogenous protein often present in the rabbit reticulocyte translation mixture. From these results, we conclude that clone pSA082 contains a cDNA insert that codes for p48 and that the identification of shorter polypeptides in the bacterial lysates indicates that the clone is not full-length. This was further confirmed by RNA blot analysis with ^{32}P-labeled pSA082 insert as probe, which identified a single mRNA species of 1.8 kb in RNA derived from human cells (Fig. 6). Primer extension using oligonucleotide primers derived from the pSA082 nucleotide sequence indicated that the clone was lacking 358 nucleotides

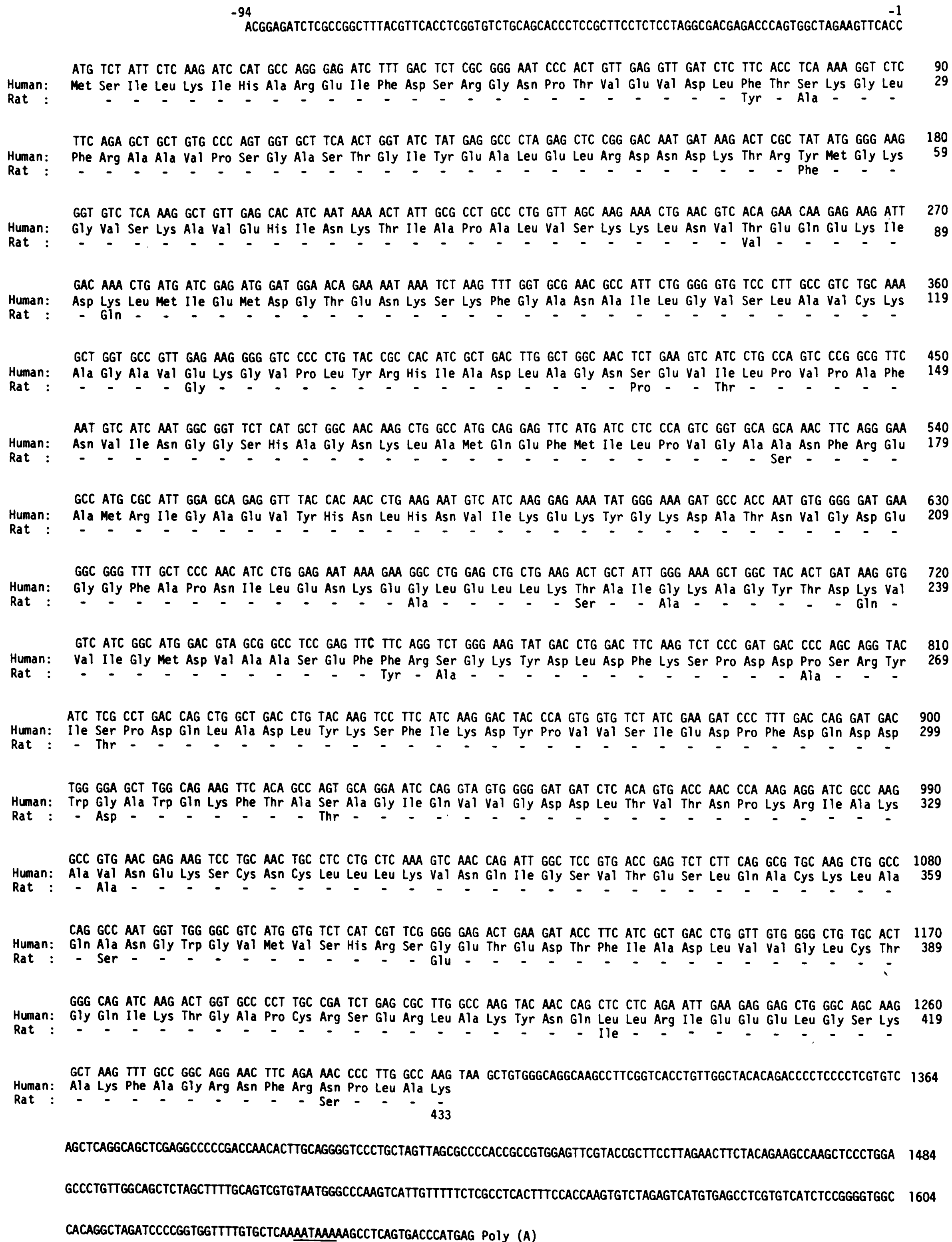

FIG. 4. Nucleotide sequence of pH48 cDNA and the predicted amino acid sequence. For comparison, the known amino acid sequence of rat non-neuronal enolase is also given (2). Dashes indicate residues that are identical in human and rat enzymes. The polyadenylylation signal in the 3′ untranslated region is underlined.

from the 5′ end (data not shown). We rescreened the cDNA library using a ^{32}P-labeled probe derived from the 5′ region of pSA082, and two hybridization-positive clones were isolated from 10,000 transformants. The size of the cDNA inserts was determined by *Pst* I cleavage, and one of them, pH48, was found to contain a 1.8-kb insert that was subsequently shown to represent the full-length mRNA of p48.

Restriction Endonuclease Map and Nucleotide Sequence of pH48. A partial restriction map and the strategy for nucleotide sequencing of pH48 are shown in Fig. 3. Clone pH48 contains an insert 1755 bp long with a continuous open reading frame of 1299 bp. The open reading frame encodes a protein of 433 amino acid residues, with a calculated molecular mass of 47,108 Da (Fig. 4). The 3′ noncoding region is 359 bp long, and a polyadenylylation signal (AATAAA) is located 20 nucleotides upstream from a poly(A)$^+$ tail. This indicates that the 3′ noncoding region is complete. The 5′ noncoding region is 94 bp long. A comparison of the predicted amino acid sequence of p48 with sequences in the Dayhoff protein data bank (National Biomedical Research Foundation, Washington, DC, 1985) revealed 67% homology with the sequence of yeast enolase (15). Since yeast enolase is 436 amino acids long, two gaps at positions 139–140 and 276 were introduced to obtain optimal alignment of the two sequences. The pH48 sequence data were then compared with the recently published sequence of a cDNA coding for the rat non-neuronal enolase (2). As illustrated in Fig. 4, the predicted amino acid sequences of rat α enolase and p48 show a very high level of homology (94%). The homology is slightly lower for the coding region at the nucleotide level (89%), with a much lower level of homology being found in 5′ and 3′ untranslated regions (41% and 20%, respectively). The high level of homology of the human sequence with the yeast and rat sequences indicates that pH48 codes for a subunit of the human enolase. Of the 24 amino acid differences between the rat and human sequences, 18 represent conservative changes. The much higher level of sequence divergence in the 3′ and 5′ noncoding regions suggests that there has been a positive pressure to maintain the enolase protein sequence through evolution. The extraordinary conservation of sequence across species is characteristic of polypeptides with multiple protein interactions, such as those which are components of multienzyme complexes. The possibility of additional interactions unrelated to its glycolytic properties has been suggested for an isomer of yeast enolase induced by heat shock (16). Preliminary experiments on human cell lines have not shown any increase in the level of enolase as a result of heat shock under conditions where the major heat shock proteins were clearly induced (data not shown). This suggests either that human cells do not contain an enolase gene that is responsive to heat shock induction or that an increase in α enolase is not required in the human heat shock response.

p48 was originally identified with an antiserum directed against the 12 carboxyl-terminal amino acids of c-myc (9). Because of its size (the amino acid sequence of c-myc predicts a 48-kDa protein) and the specificity of the reaction with the peptide antisera, p48 was identified as the c-myc protein (9). It was subsequently shown that the c-*myc* gene codes for a protein with an aberrant apparent molecular mass of 62–65 kDa on $NaDodSO_4$ gels (17). There is no significant sequence homology between enolase and c-myc, and only a limited homology can be detected to the 12 amino acid immunogen that specifically inhibits the reaction of the antipeptide serum and p48. This type of crossreaction with antibodies directed against specific epitopes has been described previously (18) and emphasizes the pitfalls of working with antipeptide sera. These types of problems may be minimized by using sera generated against several different peptides to substantiate identifications.

Enzymatic Activity. Native enolase is a dimer composed of two equal subunits held together by Mg^{2+} ions, and the dimeric structure is required for enzymatic activity (3). Since p48 was originally purified under denaturing conditions (1), the purified protein was monomeric and inactive. To show definitively that p48 is a subunit of human enolase, we have assayed enzyme activity in p48 purified from cell lysates under nondenaturing conditions. After ammonium sulfate precipitation and preparative isoelectric focusing (1), the p48-enriched fractions were pooled and the Mg^{2+} concentration was adjusted to 4 mM in order to maintain dimerization. The adjusted fractions, where p48 is already 90% pure (1), were then fractionated on a Sephadex G-150 column that had been calibrated with molecular mass markers. The eluted fractions were monitored both for enolase activity and for the presence of p48. Enzyme activity was measured by the increase in absorption at 240 nm that occurs as a result of the conversion of 2-phospho-D-glycerate to phospho*enol*pyruvate under standard assay conditions (12). As shown in Fig. 5, the absorbance peak (continuous line) corresponding to p48, as monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis and immunoblot analysis of the single fractions, overlaps the enolase activity profile (broken line). Further, the molecular mass of the eluted protein was above 80 kDa and is consistent with the size reported for the native form of human enolase dimer (19). Aliquots from G-150 fractions were also preincubated with anti-p48 antibodies, and the supernatants were tested for enzymatic activity. The immune serum sequestered essentially all the enolase activity, whereas preimmune serum did not affect the activity (data not shown).

Isoenzyme analysis (13) was also performed on p48, which was purified under nondenaturing conditions, and compared to enolase isoenzyme present in total lysates from Daudi, Jurkat, and other human cell lines. The purified protein comigrated with the slow form of enolase that is detected in these cells (data not shown), which has been identified as the α form (13). These results definitively demonstrate that p48 is the subunit of the human α enolase.

Changes in the Levels of mRNA. We have previously shown that p48 is present at relatively high levels in all actively proliferating cells but at very low levels in resting peripheral blood lymphocytes, where its synthesis is induced upon mitogenic stimulation (1). To characterize this induction, we analyzed RNAs isolated from human peripheral blood lymphocytes by blot hybridization before and after stimulation with phytohemagglutinin (PHA). Enolase-specific message

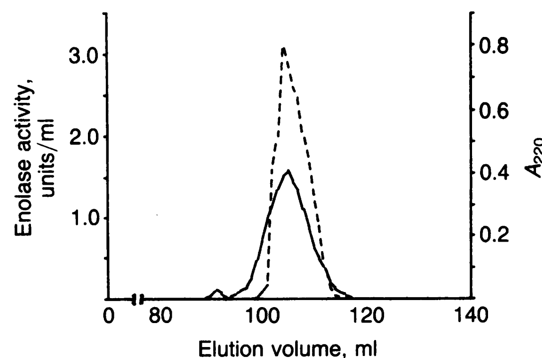

FIG. 5. Chromatographic elution and enolase activity of human p48. The pooled p48 fraction from preparative isoelectrofocusing, which was judged 90% pure by polyacrylamide gel electrophoresis, was chromatographed on a Sephadex G-150 column as described in *Materials and Methods*. Each fraction was assayed for activity (broken line) and the absorbance at 220 nm was measured (continuous line). Transferrin (74 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) were used as markers to calibrate the column.

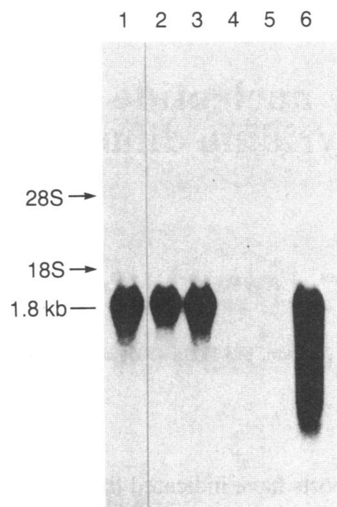

FIG. 6. Size determination and mitogenic induction of human enolase mRNA. Peripheral blood lymphocytes were isolated (1) and cultured in the absence or presence of phytohemagglutinin (10 μg/ml) for various lengths of time. Cytoplasmic RNA (20 μg) was electrophoresed, transferred to a Nytran filter, and hybridized with ^{32}P-labeled pSA082 insert. RNA was from human Burkitt lymphoma line Daudi (lane 1), human fibroblast line PAF (lane 2), or human lymphoblastoid line GM1500 (lane 3) or from human peripheral blood lymphocytes cultured without added mitogen (lane 4) or with phytohemagglutinin for 3 hr (lane 5) or 24 hr (lane 6). Positions of 28S and 18S rRNA are indicated.

was almost undetectable in resting lymphocytes (Fig. 6, lane 4) but increased to a very high level after 24 hr of mitogenic stimulation (lane 6). This level is roughly equivalent to that found in exponentially growing human cell lines (Fig. 6, lanes 1–3) independent of the cell type. A similar though less dramatic increase was found following stimulation of growth-arrested mouse 3T3 cells with 20% fetal bovine serum (data not shown). In both cases, the peak of expression was reached 12–24 hr after stimulation, and the level remained roughly constant afterward. These results are in agreement with those of Matrisian *et al.* (20), who reported the cloning of cDNAs corresponding to mRNAs specifically stimulated in quiescent rat fibroblasts by growth factors or serum and identified them as encoding five glycolytic enzymes, including enolase. Although the increase in levels of glycolytic enzymes after mitogenic stimulation may simply reflect a general growth response, glycolysis has also been suggested to have a regulatory role in the initiation of DNA synthesis (21). Several glycolytic enzymes including enolase have been shown to be substrates for tyrosine kinases produced in Rous sarcoma virus-infected cells. Although no effect on enzyme activity due to phosphorylation has been demonstrated, it is possible that phosphorylation may affect additional protein interactions not yet defined.

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