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19. Space-filling models of peptide 2 and the monomer unit of *B. pertussis* lipid A (*20*) were produced with Alchemy II software (Tripos Associates Inc, St. Louis, MO) on an HP-Vectra 486/33T 33 MHz computer and were elaborated in condition of minimal energy with cut-off 0.01.
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Sequence-Specific Binding of Transfer RNA by Glyceraldehyde-3-Phosphate Dehydrogenase

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A transfer RNA (tRNA) binding protein present in HeLa cell nuclear extracts was purified and identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Studies with mutant tRNAs indicated that GAPDH recognizes both sequence and structural features in the RNA. GAPDH discriminated between wild-type tRNA and two tRNA mutants that are defective in nuclear export, which suggests that the protein may participate in RNA export. The cofactor nicotinamide adenine dinucleotide disrupted complex formation between tRNA and GAPDH and thus may share a common binding site with the RNA. Indirect immunofluorescence experiments showed that GAPDH is present in the nucleus as well as in the cytoplasm.

The primary transcripts of eukaryotic genes are processed extensively in the nucleus, and the mature RNAs are exported to the cytoplasm. Although nuclear RNA export is fundamental to gene expression, we know relatively little about its underlying mechanisms. By analogy with nuclear protein import (*1*), the process is likely to involve nuclear proteins that bind specifically to the RNAs being exported. Consistent with this prediction, nuclear export of tRNAs as well as m^7G-capped RNAs is a saturable, carrier-mediated process (*2*, *3*). Furthermore, specific mutations within tRNA (*4*) and 5S ribosomal RNA (*5*) can block export of these RNAs.

The single base substitution G57U in human $tRNA_i^{Met}$ has been shown to reduce nuclear export of the RNA in microinjected frog oocytes (*4*). To identify proteins that participate in tRNA export, we looked for nuclear proteins that bound to wild-type $tRNA_i^{Met}$ but not to the G57U mutant. As measured by an RNA mobility-shift assay, we found such an activity in the 0.1 M KCl DEAE-Sepharose fraction of a HeLa cell nuclear extract (6). This tRNA binding activity was further fractionated, and the polypeptide composition of the chromatographic fractions was monitored by SDS–polyacrylamide gel electrophoresis (Fig. 1A). The most purified fraction, the 0.5 M KCl eluate from the polyuridylic acid [poly(U)] column, contained a single polypeptide of approximately 37 kD. Both the partially purified (6) and the homogeneous preparations of the 37-kD protein discriminated between the wild-type $tRNA_i^{Met}$ and the G57U mutant (Fig. 1B). The tRNA binding activity was heat-labile and resistant to digestion with micrococcal nuclease (6). The 37-kD polypeptide was then purified in sufficient quantity for microsequence analysis. A tryptic peptide derived from the protein was found to contain the sequence LISWYDNEFGYSNR (*7*). A database search revealed that this peptide sequence is present within the COOH-terminal region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a 37-kD glycolytic enzyme (*8*).

To determine whether tRNA binding was mediated by GAPDH, we performed an immunodepletion experiment. A binding reaction mixture containing ^{32}P-labeled tRNA and the purified 37-kD protein was incubated with either a polyclonal antibody to GAPDH or preimmune serum, and the resulting antigen-antibody complexes were removed with Pansorbin (Calbiochem, San Diego, California). The antibody to GAPDH, but not the preimmune serum, depleted the tRNA-protein complex from the supernatant (6).

A commercial preparation of human GAPDH (Sigma) also contained tRNA binding activity, and, like the 37-kD protein, bound to wild-type $tRNA_i^{Met}$ but not to the G57U mutant (Fig. 1B). Neither the 37-kD protein nor the commercial GAPDH bound to VA1 RNA, an unrelated adenoviral RNA transcribed by RNA polymerase III. Taken together (9), these data provide strong evidence that the tRNA binding activity was in fact GAPDH.

GAPDH also bound to in vitro–transcribed *Escherichia coli* $tRNA^{Tyr}$ and yeast

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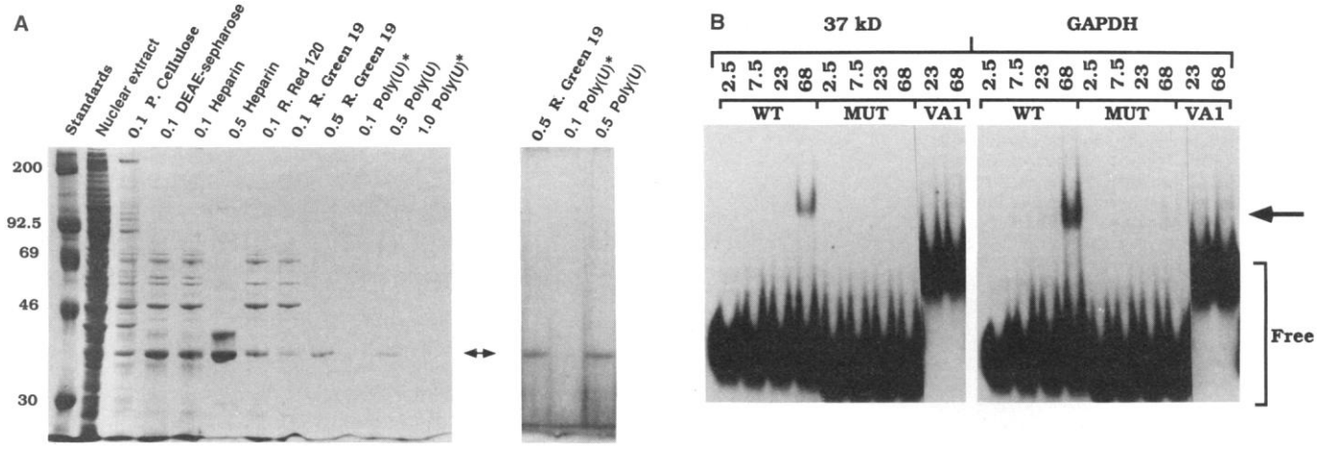

Fig. 1. Identification of a tRNA binding protein (in a HeLa cell nuclear extract) as GAPDH. (**A**) Protein profile. A crude HeLa cell nuclear extract (*12*) was fractionated through the indicated columns (*25*). The chromatographic fractions were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R250 (left) or silver nitrate (right). The 37-kD protein is indicated by an arrow and inactive fractions are indicated with an asterisk. The columns are P. Cellulose, phosphocellulose; R. Red 120, Reactive Red 120; R. Green 19, Reactive Green 19, and the numbers represent the molar KCl concentration used for elution. Molecular size markers are indicated to the left in kilodaltons. (**B**) Sequence-specific tRNA binding activity of the 37-kD protein and commercial human GAPDH (Sigma). The indicated concentrations (mM) of the 37-kD protein or GAPDH were incubated with the indicated equimolar amounts of ^{32}P-labeled wild-type $tRNA_i^{Met}$ (WT), the G57U mutant (MUT), or VA1 RNA and analyzed in an RNA mobility-shift assay on a native gel (*25*). The human GAPDH was >99% homogeneous. The positions of the free probe and tRNA-protein complex (arrow) are indicated.

$tRNA^{Ser}$ with an apparent equilibrium dissociation constant (K_d) of $\sim 1.8 \times 10^{-8}$ M (*6*). This dissociation constant is similar to that of other eukaryotic sequence-specific RNA binding proteins (*10*). The fact that GAPDH bound to in vitro–transcribed tRNAs indicates that posttranscriptional modifications are not required for recognition.

We then analyzed a series of tRNA deletion and substitution mutants to gain insight into the tRNA sequences recognized by GAPDH. Deletions of $tRNA^{Tyr}$ anticodon stem-loop (ΔAC), variable-loop (ΔV-1 and ΔV-2; Fig. 2A), anticodon stem-loop and variable-loop (ΔAV), and T stem-loop (ΔTS) substantially reduced GAPDH binding (Fig. 2A). Notably, substitution of the T-loop sequence (TL), which contains G57, also markedly decreased GAPDH binding. In the case of $tRNA^{Phe}$, a G53C;C61G mutation decreased binding of GAPDH, whereas other mutations, such as C13U;G22A, C13U;G46A, and C13U;G22A;G46A, enhanced binding affinity (Fig. 2B). GAPDH had approximately three to nine times lower affinity for $tRNA^{Phe}$ than for $tRNA^{Tyr}$ or $tRNA^{Ser}$.

We also analyzed the effects of three additional single nucleotide substitution $tRNA_i^{Met}$ mutants on GAPDH binding (Fig. 2B). The C56U mutant, which like G57U is export-defective (*4*), bound to GAPDH with lower affinity than wild-type tRNA. In contrast, the C62U mutant, which has a small effect on the rate of nuclear export (*4*), and the C61U mutant, whose rate of export

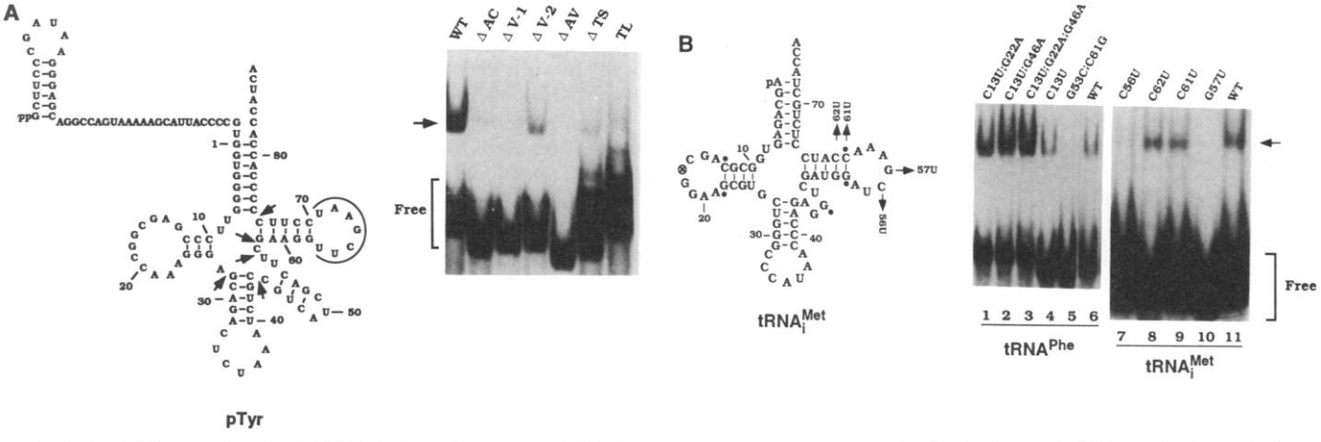

Fig. 2. Analysis of tRNA mutations for GAPDH binding. (**A**) Analysis of $tRNA^{Tyr}$ mutants. Nucleotides are numbered consecutively from the 5′ end of mature tRNA. The positions of internal deletions are indicated in the schematic of wild-type precursor $tRNA^{Tyr}$ (pTyr) by arrows. Deletions include nucleotides (nt) 28 to 44 (ΔAC), nt 45 to 56 (ΔV-1), nt 45 to 55 (ΔV-2), nt 28 to 44 and 46 to 55 (ΔAV), and nt 58 to 74 (ΔTS). The nucleotide substitution in the T-loop, nt 64 to 69, by the sequence GGAUCCG is indicated with an arc (TL). The wild-type pTyr and mutant RNAs were transcribed in vitro by T7 RNA polymerase (*26*), and the ^{32}P-labeled RNAs were incubated with GAPDH and analyzed on a native gel. (**B**) Analysis of tRNA nucleotide substitution mutants. The positions of mutations used previously for nuclear export studies (*4*) are indicated in the schematic of $tRNA_i^{Met}$ by arrows. The positions of $tRNA^{Phe}$ mutations are indicated in the same schematic by black dots (*27*). Human GAPDH (69 nM) was incubated with the indicated $tRNA^{Phe}$ (lanes 1 to 6) or $tRNA_i^{Met}$ (lanes 7 to 11) wild-type tRNA or mutant tRNAs and analyzed on a native gel. The $tRNA^{Phe}$ constructs were transcribed in vitro by T7 RNA polymerase (*26*), and the $tRNA_i^{Met}$ constructs were transcribed in a HeLa cell nuclear extract (*25*). Arrow and Free are as in Fig. 1B.

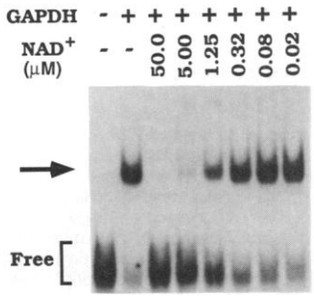

Fig. 3. Inhibition of tRNA binding by cofactor NAD^+. RNA binding reactions that contained human GAPDH (~69 nM) and ^{32}P-labeled $tRNA^{Ser}$ were incubated with the indicated concentration of NAD^+. Arrow and Free are as in Fig. 1B.

has not been determined, both bound to GAPDH with the same affinity as wild-type tRNA. These results provide evidence that GAPDH recognizes several tRNAs in a manner that most likely involves both sequence and structure.

GAPDH contains a binding site for its cofactor, nicotinamide adenine dinucleotide (NAD^+). This binding site, referred to as the Rossmann fold, is conserved among dehydrogenases (*11*). Because NAD^+ is a dinucleotide, we examined the relation between the NAD^+ binding site and the tRNA binding site. In the RNA mobility-shift assay, increasing concentrations of NAD^+ progressively disrupted the tRNA-GAPDH complex (Fig. 3). The simplest interpretation of this result is that the NAD^+ binding site is a component of the tRNA binding site. However, the concentration of NAD^+ required for 50% inhibition of tRNA binding was approximately 100-fold higher than the K_d value of GAPDH binding to tRNA. Lactate dehydrogenase, which also contains the Rossmann fold, had no detectable tRNA binding activity (6). Together, these data indicate that the conserved NAD^+ binding site constitutes only a portion of the tRNA binding domain in GAPDH.

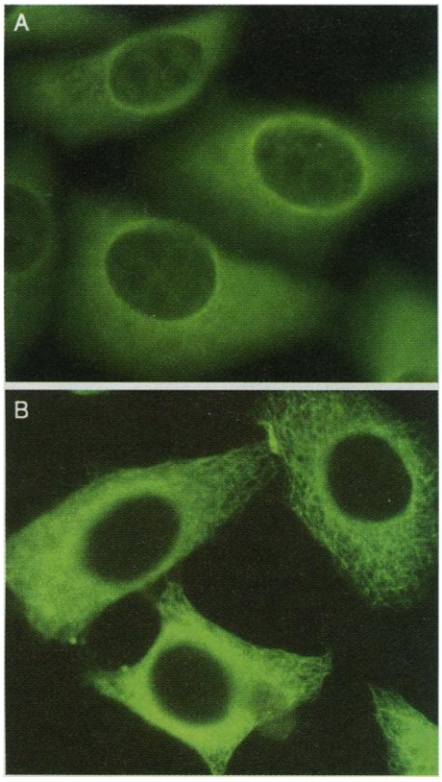

Fig. 4. Localization of GAPDH in HeLa cells by indirect immunofluorescence (*28*). (**A**) Antibody to GAPDH. (**B**) Antibody to β-tubulin.

We originally purified the 37-kD protein from a HeLa cell nuclear extract (*12*). To address the possibility that GAPDH in the nuclear extract might have resulted from cytoplasmic contamination during aqueous fractionation, we also determined the intracellular distribution of GAPDH by indirect immunofluorescence (Fig. 4). In addition to its high concentration in the cytoplasm, GAPDH was present in the nucleus with apparent exclusion of nucleoli. An antibody to β-tubulin, a cytoplasmic control, did not show detectable staining of the nucleoplasm or nucleolar exclusion. These data and previous studies (*13*) provide strong evidence that GAPDH is also localized in the nucleus.

Although the precise delineation of the tRNA elements that mediate recognition by GAPDH must await further studies, our mutational data provide insight into this issue. The tRNA mutants G57U, C56U, G53C;C61G, TL, and ΔTS showed substantially reduced affinities for GAPDH binding. The lesions in these tRNAs are all clustered in the T stem-loop region, which suggests that this is an element of recognition. However, deletion of the anticodon- and variable-loops also markedly reduced GAPDH binding. Thus, either GAPDH recognizes a region larger than the T stem-loop or these other RNA regions play a structural role, perhaps to present the core RNA recognition element appropriately. The enhanced binding affinity of some $tRNA^{Phe}$ mutants may result from the unmasking of the GAPDH binding site.

The amino acid sequence of GAPDH indicates that the RNA binding domain of GAPDH is distinct from several well-characterized RNA binding motifs, such as the ribonucleoprotein consensus domain and the arginine-rich RNA binding domain (*14*). The NAD^+ competition experiment strongly suggests that the tRNA binding domain of GAPDH includes the NAD^+ binding site (the Rossmann fold). This raises the possibility that the enzymatic activity of GAPDH can be regulated by tRNA binding or, conversely, that the RNA binding activity of GAPDH can be regulated by NAD^+. The Rossmann fold, initially discovered as a motif conserved among dehydrogenases (*11*), is also present in all class I aminoacyl-tRNA synthetases (*15*). Notably, in glutaminyl-tRNA synthetase the Rossmann fold appears to engage in a sequence-specific interaction with the acceptor stem of tRNA (*16*). Thus, this motif may function as a component of the tRNA binding domain in both class I aminoacyl-tRNA synthetases and GAPDH.

GAPDH has been implicated in several other cellular activities unrelated to glycolysis, such as binding to single-stranded nucleic acids, protein phosphorylation, interaction with microtubules, and DNA repair (*17*). Although the identification of a glycolytic enzyme as a sequence-specific RNA binding protein was unexpected, there are several other examples of multifunctional proteins involved in RNA biogenesis. First, the nuclear-encoded tyrosyl-tRNA synthetase also functions as a splicing factor for mitochondrial group I introns (*18*). Second, the IRE (iron-responsive element) RNA binding protein is homologous with and also has the enzymatic activity of aconitase, a mitochondrial citric acid cycle enzyme (*19*). Finally, the product of the yeast gene *SRN1*, which was identified as a suppressor of the RNA processing defect in a *mal-1* mutant, is identical with Hex2/Reg1, a negative regulator of glucose repression (*20*). Like our data, the latter two results suggest a link between energy metabolism and RNA biogenesis.

The observation that GAPDH is present in both the cytoplasm and nucleus (Fig. 4) (*13*) raises the possibility that it may shuttle between the two compartments. There are several known shuttle proteins, including nucleolin, B23/No38, TFIIIA, influenza M1, and hnRNP A1 (*5, 21*). Shuttle proteins are attractive candidates for the mediation of nucleocytoplasmic transport, and some of them have in fact been implicated in RNA transport (*5, 21*). Although as yet there is no direct evidence that GAPDH is involved in RNA nuclear export, our findings are consistent with and suggestive of this possibility. Genetic experiments in yeast that demonstrate that a GAPDH gene is required for viability even when lactate is the carbon source (*22*) also indicate that GAPDH performs an essential, nonglycolytic function. Our findings that GAPDH has a sequence-specific RNA binding activity and is present in the nucleus suggest that this nonglycolytic function may be nuclear and related to RNA biogenesis.

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28. HeLa cells were washed in phosphate-buffered saline (pH 7.4) and fixed in methanol and acetone at −20°C. Indirect immunofluorescence was performed with a polyclonal antibody to GAPDH (*24*) or a monoclonal antibody to β-tubulin and fluorescein-conjugated secondary antibodies. Cells were examined in a Zeiss Axiophot epifluorescence microscope and photographed with Ektachrome 400 film (Kodak).
29. We thank M. Zasloff, W. R. Folk, O. C. Uhlenbeck, Y. Yuan, and S. Altman for tRNA constructs; R. W. Allen for antibody to GAPDH; R. Singer for antibody to β-tubulin; R. Reddy for HeLa cell tRNAs and helpful discussions; O. C. Uhlenbeck for advice and encouragement; J. Valcárcel and S. Gupta for discussions; members of M.R.G.'s laboratory for comments on the manuscript; and T. O'Toole for secretarial assistance. Supported by a grant from NIH (GM35490) to M.R.G., and by a fellowship from the Leukemia Society of America to R.S.

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Tumor Rejection After Direct Costimulation of CD8$^+$ T Cells by B7-Transfected Melanoma Cells

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A variety of tumors are potentially immunogenic but do not stimulate an effective anti-tumor immune response in vivo. Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the costimulatory signals necessary for full activation of T cells. Expression of the costimulatory ligand B7 on melanoma cells was found to induce the rejection of a murine melanoma in vivo. This rejection was mediated by CD8$^+$ T cells; CD4$^+$ T cells were not required. These results suggest that B7 expression renders tumor cells capable of effective antigen presentation, leading to their eradication in vivo.

T cells recognize processed peptide antigens complexed with molecules of the major histocompatibility complex (MHC) (*1–4*). However, recognition of antigen by the T cell antigen receptor (TCR) is not sufficient for activation; a second costimulatory signal is required (*5*). The costimulation results from an interaction of the CD28 molecule on the T cell surface with its ligand, B7, on the surface of an antigen-presenting cell (APC) (*6–12*). The expression of B7 is generally limited to "professional" APCs: macrophages, dendritic cells, and activated B cells (*7, 13, 14*). Most epithelial cells do not express B7. One possibility to account for the tumorigenicity of MHC-expressing tumors is that despite presentation of potentially immunogenic peptides in the context of MHC molecules, tumors may lack the costimulatory molecule, B7, and thus fail to elicit an effective anti-tumor T cell response (*15–20*). We now show that provision of costimulatory activity can lead to tumor rejection in vivo, and that this strategy bypasses the need for exogenous help from CD4$^+$ T cells by directly activating CD8$^+$ T cells.

The murine melanoma cell line K1735 expresses both MHC class I and class II molecules and stimulates a specific, but ineffective, immune response in vivo (*21, 22*). K1735 cells did not provide costimulation to T cells in a standard in vitro costimulation assay (below). In initial experiments to test the importance of costimulation in the generation of an anti-tumor response, monoclonal antibody (MAb) to CD28 was used as an exogenous source of costimulatory activity (*23*). Fragments of K1735 solid tumors raised in athymic mice were implanted subcutaneously (s.c.) into syngeneic C3H/HeN mice. The mice were treated with repeated intraperitoneal injections of MAb to CD28 (ascites). Treatment with anti-CD28 slowed the growth of the melanoma (Fig. 1). Although complete

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