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Anticodon Switching Changes the Identity of Methionine and Valine Transfer RNAs

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The anticodon has previously been shown to play a role in recognition of certain transfer RNAs by aminoacyl-tRNA synthetases; however, the extent to which this sequence dictates tRNA identity is generally unknown. To investigate the contribution of the anticodon to the identity of *Escherichia coli* methionine and valine tRNAs, in vitro transcripts of these tRNAs were prepared that contained normal and interchanged anticodon sequences. Transcripts containing wild-type tRNA sequences were excellent substrates for their respective cognate aminoacyl-tRNA synthetases and were effectively discriminated against by a variety of noncognate enzymes. The mutant tRNAs produced by switching the anticodon sequences lost their original tRNA identity and assumed an identity corresponding to the acquired anticodon sequence. These results indicate that the anticodon contains sufficient information to distinguish methionine and valine tRNAs with high fidelity.

Attachment of the appropriate amino acid to each tRNA molecule is a crucial step in the translation of genetic information. The highly accurate aminoacylation of tRNAs is catalyzed by aminoacyl-tRNA synthetase enzymes specific for each of the 20 amino acids. The amino acid acceptor specificity, or identity, of each tRNA is determined by a particular set of structural features that allows recognition by one synthetase and excludes recognition by all others. The location of these identity elements remains largely unknown (*1*); however, significant progress has recently been made in determining structural features important for recognition of several tRNAs, including *Escherichia coli* alanine (*2, 3*), glutamine (*4*), phenylalanine (*5*), and serine (*6*) tRNAs and yeast $tRNA^{Phe}$ (*7, 8*). In addition, it has been known for some time that important recognition sites for aminoacyl-tRNA synthetases are present in the anticodons of certain tRNAs (*9*). Single base changes in the anticodon sequences of *E. coli* $tRNA_f^{Met}$ (recognizing RNA encoding the initiator methionine) (*10, 11*), $tRNA^{Trp}$ (*12*), $tRNA^{Arg}$ (*13*), and $tRNA^{Gly}$ (*14*), yeast $tRNA^{Val}$ (*15*), and beef $tRNA^{Trp}$ (*16*) have dramatic effects on aminoacylation of these tRNAs by their cognate synthetases. In addition, the specificity of amino acid acceptance of *E. coli* $tRNA^{Trp}$ (*12, 17*), *E. coli* $tRNA_f^{Met}$ (*18*), and yeast $tRNA^{Tyr}$ (*19*) is affected by alterations in the anticodon sequence. In only a few cases, however, has the quantitative contribution of the anticodon to tRNA identity been evaluated.

The CAU anticodon of methionine tRNAs (recognizing the codon AUG) is a crucial site of interaction with *E. coli* methionyl-tRNA synthetase (*10, 11*). To determine the extent to which the anticodon determines the identity of $tRNA^{Met}$, we have now transferred the methionine anticodon to a valine tRNA and examined the amino acid acceptor activity of the mutant $tRNA^{Val}$. In addition, we have prepared a methionine tRNA containing a valine anticodon and determined its aminoacylation specificity.

Wild-type and mutant methionine and valine tRNAs were prepared by in vitro transcription with T7 RNA polymerase from plasmids containing the appropriate tRNA genes, according to the methodology recently described by Sampson and Uhlenbeck (*7*). The genes were constructed from synthetic DNA oligonucleotides joined to a T7 RNA polymerase promoter; the promoter was positioned to initiate transcription with the 5′ terminal nucleotide of the mature tRNA. A Bst NI restriction enzyme site was included at the 3′ terminus of the gene to allow generation of a 3′ CCA end on the mature tRNA after run-off transcription from Bst NI–digested plasmid DNA. Normal 5′ phosphate termini were produced by inclusion of excess 5′ mononucleotide corresponding to the initiating nucleotide in the transcription reaction mixture (*7*). The in vitro transcripts obtained from plasmids carrying the genes for the elongator methionine tRNA ($tRNA_m^{Met}$) and for the major species of valine tRNA($tRNA_1^{Val}$) contain none of the modified bases normally found in the native tRNAs, but showed near normal amino acid acceptor activity with cognate aminoacyl-tRNA synthetases (Tables 1 and 2).

The anticodons of the methionine and valine tRNAs contain the same nucleotide bases in inverted order. Wild-type $tRNA^{Val}$ (anticodon UAC) is an extremely poor substrate for methionyl-tRNA synthetase. At high tRNA concentrations, the initial rate of methionine acceptance increases linearly with increasing $tRNA^{Val}$ (UAC) up to 40 μM, indicating that this concentration is far

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below the K_m (Michaelis constant) for the tRNA (*20*). Under these conditions, accurate values for the kinetic parameters for aminoacylation with methionine cannot be obtained; however, the slope of the linear plot of initial rate versus tRNA concentration gives V/K_m. Comparison of V/K_m for the transcripts of $tRNA_m^{Met}$ (CAU) and $tRNA^{Val}$ (UAC) shows that the relative specificity of methionyl-tRNA synthetase for the cognate tRNA is nearly six orders of magnitude greater than that for wild-type $tRNA^{Val}$ (Table 1). Substitution of the valine UAC anticodon with the methionine CAU anticodon converted $tRNA^{Val}$ into a substrate having wild-type methionine acceptor activity (Table 1), demonstrating the importance of the anticodon sequence in discrimination of tRNA substrates by methionyl-tRNA synthetase.

The question remains as to whether the methionine and valine tRNAs share any common structural features outside of the anticodon region that contribute to $tRNA^{Met}$ identity. Any such structural features must also be shared by other tRNAs that are efficient substrates for *E. coli* methionyl-tRNA synthetase. These include initiator tRNAs from a wide variety of sources and elongator methionine tRNAs from prokaryotes and the organelles of higher organisms (*21–23*). Comparisons of the sequences of these methionine tRNAs reveals that they contain identical nucleotides at 15 sites in the tRNA structure. Ten of these nucleotides are also common to tRNAs specific for many other amino acids. Five nucleotides are uniquely common to tRNAs that are good substrates for the *E. coli* methionine enzyme, including the three anticodon bases and the base pair C3·G70 in the acceptor stem. The $tRNA^{Val}$ (CAU) contains a G3·C70 sequence, thus this site is eliminated as an identity element for $tRNA^{Met}$. Figure 1 shows the remaining nucleotides that are present in all tRNAs shown to be efficient substrates for *E. coli* methionyl-tRNA synthetase. Only the anticodon nucleotides are uniquely common to this set of tRNAs.

Previous studies with anticodon derivatives of the native *E. coli* initiator methionine tRNA showed that C34 at the wobble position of the anticodon is the most important nucleotide for recognition of $tRNA_f^{Met}$ by the methionine synthetase (*11*). Similar results were observed with transcripts of the elongator methionine tRNA (Table 1). Sub-

Table 1. Kinetic parameters for aminoacylation of tRNAs with *E. coli* methionyl-tRNA synthetase. Genes for methionine and valine tRNAs were constructed from synthetic deoxyoligonucleotides (*6*). The sequences were based on those previously reported for the *E. coli* elongator methionine tRNA (*27*) and the major valine isoacceptor, $tRNA^{Val}$ (*28*). The genes were joined to a synthetic T7 RNA polymerase promoter, inserted into the phagemid vector pUC119, and cloned in *E. coli* strain JM109 (*29*). Transcripts of the tRNA genes were prepared in reaction mixtures containing Bst NI–cut plasmid DNA (0.5 mg/ml), 40 m*M* tris-HCl, *p*H 8, 5 m*M* dithiothreitol, 2 m*M* spermidine, 25 m*M* NaCl, 22 m*M* $MgCl_2$, 4 m*M* each nucleotide 5′ triphosphate (*p*H 8), 20 m*M* guanosine 5′ phosphate, and pure T7 RNA polymerase (280 μg/ml). Transcripts were purified by polyacrylamide gel electrophoresis followed by high-performance liquid chromatography (*30*). Specific activities for amino acid acceptance were 1100 to 1300 pmol per unit of absorbance at 260 nm (pmol/A_{260}). Native $tRNA_f^{Met}$ (1700 pmol/A_{260}) was purchased from Boehringer and native $tRNA_m^{Met}$ (1100 pmol/A_{260}) was purified from crude *E. coli* K-12 tRNA. Reaction mixtures for assay of methionine acceptance contained 20 m*M* imidazole-HCl, *p*H 7.5, 0.1 m*M* EDTA, 2 m*M*,adenosine triphosphate (ATP), 150 m*M* NH_4Cl, bovine serum albumin (10 μg/ml), 0.5 to 50 μ*M* tRNA, 17 μ*M* [^{35}S]methionine (2000 to 20,000 cpm/pmol) and magnesium chloride as follows: native $tRNA_f^{Met}$, 4 m*M*; native $tRNA_m^{Met}$, 5 m*M*; $tRNA_m^{Met}$ (CAU), 8 m*M*; $tRNA^{Val}$ (CAU), 10 m*M*; $tRNA_m^{Met}$ (UAU), 8 m*M*; $tRNA_m^{Met}$ (GAU), 8 m*M*; $tRNA^{Val}$ (UAC), 10 m*M*. Samples were incubated at 37°C with purified methionyl-tRNA synthetase and treated as described (*11*). The enzyme used for these assays had been stored at −20°C for a prolonged period and had a turnover number (5 s^{-1}) at saturating $tRNA_f^{Met}$ approximately threefold lower than that of freshly prepared enzyme under the same conditions. Reactions were not carried out at saturating amino acid concentrations, thus apparent K_m values are reported. Individual kinetic parameters could not be measured for $tRNA_m^{Met}$ (UAU), $tRNA_m^{Met}$ (GAU), and $tRNA^{Val}$ (UAC). V/K_m was determined as described in the text.

tRNA	Apparent K_m (μ*M*)	V (μmol min^{-1} mg^{-1})	V/K_m	Relative V/K_m
Native $tRNA_f^{Met}$	0.6	0.8	1.3	7×10^5
Native $tRNA_m^{Met}$	0.6	2.2	3.7	2×10^6
$tRNA_m^{Met}$ (CAU)	1.1	1.9	1.7	9×10^5
$tRNA^{Val}$ (CAU)	1.2	1.5	1.3	7×10^5
$tRNA_m^{Met}$ (UAU)			1.7×10^{-4}	85
$tRNA_m^{Met}$ (GAU)			1.5×10^{-5}	7.5
$tRNA^{Val}$ (UAC)			2×10^{-6}	1

Table 2. Kinetic parameters for aminoacylation of tRNAs with *E. coli* valyl-tRNA synthetase. *E. coli* valyl-tRNA synthetase was partially purified (*31*) from an overproducing strain (*32*) and contained no detectable methionyl-tRNA synthetase. Native $tRNA_1^{Val}$ (1200 pmol/A_{260}) was purchased from Boehringer. Transcripts were prepared and kinetic parameters determined as described in the legend to Table 1. Aminoacylation reactions contained 0.1*M* tris-HCl, *p*H 7.5, 10 m*M* KCl, 2 m*M* ATP, 50 μ*M* [^{14}C]valine (390 cpm/pmol), bovine serum albumin (100 μg/ml) 0.5 to 50 μ*M* tRNA, and magnesium chloride as follows: native $tRNA_1^{Val}$, 5 m*M*; $tRNA^{Val}$ (UAC), 11 m*M*; all $tRNA_m^{Met}$ derivatives, 5 m*M*.

tRNA	Apparent K_m (μ*M*)	V (μmol min^{-1} mg^{-1})	V/K_m	Relative V/K_m
Native $tRNA_1^{Val}$	0.5	2.0	4.0	5×10^5
$tRNA^{Val}$ (UAC)	1.0	1.5	1.5	2×10^5
$tRNA_m^{Met}$ (UAC)	5.0	0.8	0.16	2×10^4
$tRNA_m^{Met}$ (UAU)			5.5×10^{-5}	7
$tRNA_m^{Met}$ (CAU)			8×10^{-6}	1

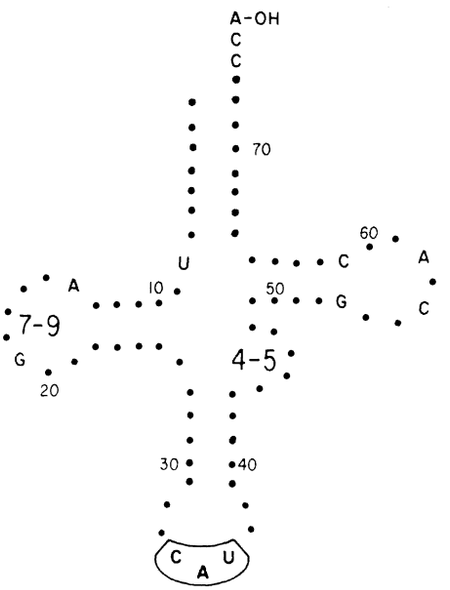

Fig. 1. Composite structure of sequences found in tRNAs efficiently aminoacylated by *E. coli* methionyl-tRNA synthetase (*21–23*). Letters indicate the positions of conserved bases. Bases uniquely conserved in methionine-accepting tRNAs are boxed. The remaining bases in the composite are common to tRNAs specific for many other amino acids. Dots indicate positions of sequence variation. Base modifications have been ignored in compiling the conserved sites. Numbering is from the 5′ end of the tRNA as described (*26*). The anticodon of native $tRNA_m^{Met}$ contains N^4-acetylcytidine at position 34. This base modification has no effect on the kinetics of aminoacylation of $tRNA_m^{Met}$ (*38*). The size of the D loop can vary from seven to nine nucleotides and the variable loop can contain either four or five nucleotides. In addition, a base pair is not required at position 1·72 and several other internal sites.

stitution of the C wobble base in $tRNA_m^{Met}$ with U or G results in a reduction in V/K_m for methionine acceptance by four and five orders of magnitude, respectively. Thus, a single nucleotide confers much of the specificity for recognition of tRNAs by methionyl tRNA synthetase. A combination of "nonmethionine" nucleotides at the other two positions of the anticodon is also sufficient to prevent aminoacylation of tRNAs by this enzyme, however. For example, native $tRNA^{Trp}$ (anticodon CCA) is not significantly mischarged with methionine, whereas a $tRNA^{Trp}$ derivative containing the CAU anticodon is an efficient substrate for the methionine enzyme (*20*). Additional negative interactions between the synthetase and sites outside of the anticodon may also assist in discrimination against noncognate tRNAs containing C34.

Little information is available about the structural features that determine the identity of *E. coli* valine tRNAs; however, an amber-suppressor derivative of $tRNA^{Val}$ has been found to insert lysine rather than valine in vivo, indicating an important role for the anticodon in the identity of this tRNA (*24*). As seen in Table 2, substitution of the valine anticodon UAC for the methionine anticodon CAU converted $tRNA_m^{Met}$ into an efficient substrate for the *E. coli* valine synthetase, increasing V/K_m by a factor of 2×10^4. Comparison of V/K_m for the $tRNA_m^{Met}$ anticodon mutants UAU and UAC shows that the presence of C36 at the 3′ end of the anticodon confers most of the specificity for valine acceptance to $tRNA_m^{Met}$. Valine and methionine tRNAs have a common base A35 in the middle position of the anticodon, which may also contribute significantly to $tRNA^{Val}$ identity. The nucleotide at the wobble position of valine tRNAs varies in different isoacceptor tRNA species in order to allow translation of the four GU*N* valine codons, thus this site may add little or no specificity to the interaction with the valine enzyme.

The $tRNA_m^{Met}$ (UAC) derivative was an approximately tenfold poorer substrate for the *E. coli* valyl-tRNA synthetase than $tRNA^{Val}$ (UAC) (Table 2). At the present time, it is not clear whether additional nucleotides outside of the anticodon make some contribution to $tRNA^{Val}$ identity or whether $tRNA_m^{Met}$ (UAC) contains one or more structural features that negatively affect its interaction with valyl-tRNA synthetase.

Examination of the methionine acceptor activity of the UAC anticodon derivative of $tRNA_m^{Met}$ reveals that it is no longer an effective substrate for methionyl-tRNA synthetase (Table 3). V/K_m for acceptance of methionine by $tRNA_m^{Met}$ (UAC) was similar to that observed with $tRNA^{Val}$ and other noncognate tRNAs. This was expected since the $tRNA_m^{Met}$ (UAU) transcript and native $tRNA_f^{Met}$ containing U34 at the wobble position are both poor substrates (Table 1) (*25*) and $tRNA_f^{Met}$ containing C36 at the 3′ end of the anticodon is also poorly recognized by methionyl-tRNA synthetase (*11*). The alteration of the methionine anticodon from CAU to UAC thus completely switched the identity of $tRNA_m^{Met}$ from methionine to valine. Similarly, $tRNA^{Val}$ containing the methionine anticodon lost its ability to be recognized by valyl-tRNA synthetase (Table 3) while assuming wild-type methionine acceptor activity (Table 1).

The fidelity of aminoacylation of the in vitro tRNA transcripts is similar to that seen with native tRNAs, suggesting that the minor bases normally present do not contribute significantly to tRNA identity. To determine whether the mutant $tRNA^{Met}$ and $tRNA^{Val}$ derivatives have relaxed specificity for aminoacylation by enzymes specific for other amino acids, we have examined the ability of five noncognate aminoacyl-tRNA synthetases to mischarge these tRNAs in vitro (Table 3). Three of the enzymes chosen for study (isoleucine, lysine, and phenylalanine) recognize cognate tRNAs of the same structural class as the methionine and valine tRNAs, and all of the enzymes normally aminoacylate tRNAs with small variable loops. Cognate tRNAs for the glutamine and glutamate synthetases are also structurally similar, but contain three rather than four base pairs in the D stem. The anticodon is known to be important for recognition of tRNA substrates by the synthetase specific for glutamine (*12, 18*), and additional data suggest that the anticodon may also be involved in the selection of cognate tRNAs by the glutamate, isoleucine, lysine, and phenylalanine enzymes (*9, 24*). Each of these noncognate synthetases discriminated well against both the wild-type and mutant methionine and valine tRNAs (Table 3), indicating that the switch in tRNA identity resulting from the interchange of the CAU and UAC anticodon sequences was specific. It will be necessary to carry out in vivo experiments to examine the fidelity of aminoacylation of the mutant tRNAs in *E. coli* cells.

Table 3. Relative V/K_m for aminoacylation of tRNA transcripts with cognate and noncognate *E. coli* aminoacyl-tRNA synthetases. V/K_m values for the purified transcipts are expressed relative to V/K_m values obtained for each aminoacyl-tRNA synthetase with the following purified cognate tRNAs: $tRNA_f^{Met}$, $tRNA_i^{Val}$, $tRNA_2^{Gln}$, $tRNA^{Glu}$, $tRNA_i^{Ile}$, $tRNA^{Lys}$, and $tRNA^{Phe}$. The synthetases for methionine and valine were obtained from *E. coli* strains overproducing these enzymes (*32, 33*) as described in the legends to Tables 1 and 2. Purified synthetases for glutamine, glutamate, and phenylalanine were generous gifts from D. Söll, J. LaPointe, and E. Holler and had turnover numbers of 0.8, 0.6, and 4 s^{-1}, respectively, at saturating concentrations of cognate tRNA as the time these assays were performed. Isoleucyl-tRNA synthetase (k_{cat} = 2 s^{-1}) was purified from *E. coli* strain CSR 603 (*34*). Partially purified lysyl-tRNA synthetase (V = 1.9 μmol min^{-1} mg^{-1} at saturating $tRNA^{Lys}$) was obtained from F. Midelfort. These enzyme preparations contained no detectable methionyl- or valyl-tRNA synthetase. Aminoacylation reactions were as described (*11, 18, 31, 34–37*).

tRNA	Aminoacyl-tRNA synthetase						
	Met	Val	Gln	Glu	Ile	Lys	Phe
$tRNA_m^{Met}$ (CAU)	1.3	2×10^{-6}	10^{-7}	$<10^{-6}$	$<10^{-7}$	$<10^{-6}$	$<10^{-7}$
$tRNA_m^{Met}$ (UAC)	10^{-7}	0.04	10^{-7}	$<10^{-6}$	$<10^{-7}$	$<10^{-6}$	$<10^{-7}$
$tRNA^{Val}$ (CAU)	1.0	10^{-5}	10^{-7}	$<10^{-6}$	$<10^{-7}$	$<10^{-6}$	$<10^{-7}$
$tRNA^{Val}$ (UAC)	1.5×10^{-6}	0.4	10^{-7}	$<10^{-6}$	$<10^{-7}$	$<10^{-6}$	$<10^{-7}$

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Cryopreservation, Culture, and Transplantation of Human Fetal Mesencephalic Tissue into Monkeys

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Studies in animals suggest that fetal neural grafts might restore lost neurological function in Parkinson's disease. In monkeys, such grafts survive for many months and reverse signs of parkinsonism, without attendant graft rejection. The successful and reliable application of a similar transplantation procedure to human patients, however, will require neural tissue obtained from human fetal cadavers, with demonstrated cellular identity, viability, and biological safety. In this report, human fetal neural tissue was successfully grafted into the brains of monkeys. Neural tissue was collected from human fetal cadavers after 9 to 12 weeks of gestation and cryopreserved in liquid nitrogen. Viability after up to 2 months of storage was demonstrated by cell culture and by transplantation into monkeys. Cryopreservation and storage of human fetal neural tissue would allow formation of a tissue bank. The stored cells could then be specifically tested to assure their cellular identity, viability, and bacteriological and virological safety before clinical use. The capacity to collect and maintain viable human fetal neural tissue would also facilitate research efforts to understand the development and function of the human brain and provide opportunities to study neurological diseases.

TRANSPLANTATION OF FETAL NEURAL tissue in rodents has been shown to alter host neurological, endocrine, cognitive, and motor functions; the alterations have been correlated with evidence of neuronal survival, axonal integration, synapse formation, appropriate neurochemical release, and electrophysiological interactions with host neurons (*1*). These studies raised the possibility that neural transplantation might benefit patients with neurological disorders, especially the neurodegenerative disorder Parkinson's disease, which involves progressive loss of striatal dopamine function (*2*). However, poor neural graft survival in monkeys (*3*) and ethical concerns about the use of human fetal tissue led instead to clinical trials with adrenal medullary autografts as a possible alternative source of replacement dopamine (*4*). The effectiveness of these adrenal autografts in patients with Parkinson's disease cannot yet be fully evaluated. Published reports are mixed, and a number of complications and problems appear to be emerging (*5*). Evidence of survival of identifiable adrenal tissue also is very limited (*6*).

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In contrast, survival and integration of transplanted monkey fetal substantia nigra (SN) tissue have been demonstrated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–treated monkeys, which have parkinsonian symptoms (*7*). After grafts were placed into regions of the caudate nucleus, there was improvement in behavioral and motor function and diminished dopamine-associated biochemical abnormalities in the regions of graft innervation. Persistent dopamine abnormalities in regions of caudate away from the grafts and in the substantia nigra (at an even greater distance away) suggest that recovery of host dopaminergic neurons is unlikely to be responsible for the behavioral and biochemical improvements. Control animals, in which grafts were placed outside the striatum or that had grafts of non–dopamine producing cells showed continued functional and biochemical abnormalities. Transplantation of fetal neural tissue, therefore, could probably replace lost function in parkinsonian patients, to the extent that MPTP-induced parkinsonism in monkeys is a model for the idiopathic human disease.

The use of fetal neural tissue from and for humans raises scientific, ethical, legal, and safety questions that are not confronted in studies of animals: (i) Can viable mesencephalic neural tissue be collected from human fetal cadavers in a manner that conforms to legal guidelines and considers ethical concerns? (ii) Can this tissue be safely implanted with appropriate confirmation of tissue and neuronal identity as well as bacteriological and virological safety? (iii) Can tissue collection and neurosurgical implantation be scheduled to minimize recipient risk while increasing the probable viability of implanted tissue? (iv) Is there evidence of graft failure or host rejection after transplantation?

Physicians in Sweden, Mexico, England, and Spain have now reported preliminary results of clinical attempts at direct transplantation of human fetal tissue into patients with Parkinson's disease (*8, 9*). The details of timing of neural tissue collection, holding or preservation methods, and evidence of cell viability in humans have not been clear in most of these studies. Collections have been made from spontaneous and elective abortions at various gestational intervals, and tissues may not have been held long enough to demonstrate specific tissue viability or biological safety. The absence of procedural details related to the use of spontaneously aborted fetuses, such as definitions of death and the necessity for rapid scheduling of transplantation procedures, raises questions about tissue viability. The two collaborating Swedish research teams with