ELIZABETH NOLAN: So where we left off last time, we were talking about using antibiotics as tools to study the ribosome. And recall that antibiotics have many different structures, can bind to the ribosome at different places. And we closed with talking about this antibiotic, puromycin, that can bind to the A-site and cause chain termination, and also molecules that are derivatives of puromycin, such as that more elaborate one with a C75 there.

And so the example of a system where puromycin has been employed, and this is just one of many, many examples, but also gives us a little new information about players in translation, involves studies of elongation factor peak, so EFP. And if you recall, where I closed last time was with the comment that this EFP over the years was implicated in a variety of cellular processes. But its precise function remained unclear. And so Rodnina and co-workers conducted a series of experiments to ask, what is the effect of EFP on peptide bond formation when different dipeptides are in the P-site? OK?

And their experiments were motivated by the fact that there was some preliminary work out there suggesting that EFP accelerates peptide bond formation, but really, the details were unclear. So we're going to look at the experiment, their initial experiment they did, which led to some new understanding about how EFP affects the translation process. So what is it that they want to do in this experiment effectively? Imagine we have our ribosome, and we have our three sites, OK?

And so what they do in this experiment is they have a dipeptide loaded in the P-site, OK, where x is some amino acid. OK, and then what they want to do is have puromycin in the A-site and then effectively monitor for peptide bond formation with or without EFP added such that the product is effectively a tripeptide, where we have fMat, the amino acid and puromycin, OK?

And keep in mind, if this is what's being monitored, there needs to be a step to hydrolyze this tripeptide off the tRNA that's in the P-site, OK? And throughout this work, how they monitored

this is that they have a radio label on the formal methionine. So you can imagine that you can somehow separate and see the dipeptide as well as this tripeptide-like molecule with the puromycin attached.

So how to set up an experiment to test this? So they do a stop-flow experiment, so you heard some more about that method in recitation last week. And so in thinking about this, we need to think about what will be mixed. So what are the components of each syringe? How will this reaction be quenched? And so beginning to think about that, the question is, how do we even get the ribosome we need to start with in order to see the reaction? Right?

So imagine that the goal is to have a pre-translocation ribosome, so effectively that dipeptide is in the P-site, and the A-site's empty. And then that assembled post-translocation ribosome needs to be mixed with puromycin such that puromycin can enter the A-site and peptide bond formation can occur. OK? So there's quite a bit of work that needs to happen to even get this experiment set up, because somehow that post-translocational ribosome needs to be made.

OK, so if we think about this from the standpoint of the experiment and using the stop-flow to rapidly mix, we have syringe 1, and we have syringe 2, and we have our mixer. OK, so what are we going to put in syringe 1? OK, so here, we're going to have the post-translocation ribosome. The A-site is empty, and the P-site holds the dipeptide attached to the tRNA.

And then in syringe 2, we're going to have puromycin here. OK, so before we get to EFP, I'm thinking about how we're going to look at that in this reaction and what it does. How are we going to get here? So what needs to be done to get this post-translocational ribosome? Is it in the sigma catalog? Bio rad? No way! And even if it were, you would be broke needing to purchase enough to do this experiment, right? You talked about needing high concentrations in recitation last week for these types of experiments.

So where does this come from? What you need to do before even getting this into your syringe here, to do a rapid mixing experiment?

AUDIENCE: You have to isolate it from cells?

ELIZABETH NOLAN: OK, so what is the likelihood of isolating-- well, what's it, what do you need to isolate from cells?

AUDIENCE: Well, you're going to need to modify it afterwards because there'll be all sorts of other things.

ELIZABETH NOLAN: Right, but what's it?

AUDIENCE: The ribosome.

ELIZABETH NOLAN: OK, so we need a ribosome. Right? What else do we need? So we need the ribosome, and we need to get this into the P-site. So how are we going to get that dipeptidyl tRNA into the P-site?

AUDIENCE: You need an mRNA.

ELIZABETH NOLAN: We need an mRNA, and we're going to design that mRNA based on what amino acids we're interested in. So we need to come up with an mRNA. What else do we need? So think back to the whole cycle.

AUDIENCE: You need EF-Tu, GTP. You need everything necessary to form the fMat to x-peptide.

ELIZABETH NOLAN: Yeah. So what does that mean first? And when does that bond form? That's the next thing, right? So can we deliver this species to the P-site, based on what we understand about translation in the past four or five lectures? No. Right? So first, the initiation complex needs to be prepared in lab, which means you need initiation factors, a ribosome, mRNA, the initiator tRNA.

And then that initiation complex needs to be purified, which is done by a type of sucrose gradient centrifugation. OK, and then what? Once that initiation complex is formed, there needs to be a round of elongation, where the ternary complex of EF-Tu. The amino acid and GTP comes in to deliver that x-tRNA x to this A-site, and then have peptide bond formation occur. OK?

And then, we also need the help of EFG to move that to the P-site, right? So that whole cycle we've talked about from a fundamental perspective needs to be done at the bench in order to get here. So there's a lot of factors that need to be purified and obtained, quite a bit of effort to just even set this experiment up. OK? So always think about where these things come from.

So we have this. We have puromycin, right? And then we want to look at the effective EFP. So the idea is, are there differences in peptide bond formation? Is it accelerated in the presence of EFP, as how some of this preliminary data indicated? And if so, is that for all amino acids? Or is it specific for certain amino acids, right? So we need to include EFP. And in these experiments, it was either omitted or included in each syringe.

And something just to think about when thinking about these rapid mixing experiments is what happens in the mixer, right? If you're having the same volume, which is the case coming from syringe 1 and 2, you're going to have a dilution in here of all of the components. Right? So these are going to be rapidly mixed in the absence or presence of EFP. There'll be some time to allow for reaction to occur.

And then, in this case, the reaction is going to be quenched. So it's the quench flow-type setup that came up in the recitation notes from last week. So in this case, we're going to have a syringe 3 with a quencher. And in this particular work, they used base, so sometimes it's acid. Sometimes it's base. And this was a solution of KOH. OK, so then after some time, OK, we can have the reaction quench.

OK, and then there'll be some sort of workup and product analysis. OK? So in this case, they chose to hydrolyse the peptidyl tRNA's and look at the peptide fragments. So you can imagine you need a method that's going to separate fMet x, whatever amino acid x is, from that product there. And then the radio label on the fMat is used for quantification.

So what happens here? And I'll just give a summary, and then we'll look at it in more detail. So what they did in these experiments-- and recall that JoAnne talked about in recitation last week, when doing these kinetic experiments, you have to tweak them quite a bit to get the exact good conditions to observe what you want to see. So imagine that happened. We have our k observed, and I'm going to show these on a log scale.

So always keep in mind, paying attention to what type of scale the axes are in. And so what we're going to look at is the k observed for formation of this tripeptide, depending on amino acid. And I'm going to generalize a bunch of the data here, and then we'll look at all the individual cases. OK? So here, we have x does not equal proline OK, and here, not colored in, is no EFP.

And shaded is k observed for the reactions conducted in the presence of EFP. OK? So what was observed in these studies, looking at having many different amino acids here? With that, many of these amino acids showed negligible difference, whether or not EFP was included in the reaction. OK? And we can look at that data in more detail from the paper on the slide.

What was very striking about these initial experiments was what happened in this case, when x equals proline here. So effectively, what they observed in this case was about 90-fold rate

acceleration. Effectively, if we compare the k observed for peptide bond formation in the absence of EFP, we see it's significantly diminished for proline if EFP isn't there.

And along those lines, it was known before that proline attached to its tRNA is a poorly reactive tRNA. So different aminoacyl tRNA's react differently in the ribosome. So there's that layer of complexity we haven't really talked about in this class yet here. So if we take a look at all these different examples, this one is the outlier. OK?

So what these data indicated is that EFP has some special role in accelerating peptide bond formation for peptide bonds that contain a C-terminal proline residue here for that. And so these experiments were just a starting point for many additional experiments that ended up showing EFP is really critical for helping the ribosome translate sequences that have consecutive prolines in a row.

So either three prolines or maybe a PPG sequence here. And in the absence of EFP, what can happen is that the ribosome stalls. So these aminoacyl tRNA's are not very reactive, and the ribosome just gets kind of stuck. And you can imagine that's not good for the cell. And then if we bring these observations back around to some of these early works that were suggesting EFP has a role in a diversity of different cellular processes, what might we ask?

We might ask, well, where do the sequences of multiple prolines come up? So what types of proteins have three prolines in a row some place in their sequence? Or something like PPG. And so they took a look at that. And if we think about E. coli, there's about 4,000 different proteins, and there's a subset of around 270 that have these types of sequences in them. So not hugely common, but they exist.

And so then ask, what do these proteins do? Right? Provided a function is known. And so what we see is within that subset of about 270 proteins, there's examples of proteins that are involved in regulation, in metabolism, you know, important cellular processes. So you can begin to understand why it might be that this protein got implicated in all these different types of phenomena, right?

But in terms of the details, it's really back here in terms of how this translation factor is helping the ribosome make a certain subset of peptide bonds there. So if you're curious about this, the paper's really wonderful. There's a number of additional interesting experiments that are done and additional methods to these kinetics there. I'm happy to point you in that direction.

So yes?

AUDIENCE: Does this rate of the reaction affect upon ribosome folding?

ELIZABETH NOLAN: It could. I mean, basically, you're talking about what happens as the polypeptide extrudes from the ribosome, right? And if you're stalled and have some piece of this nascent polypeptide on the outside. Ribosomes stalling, yeah, what does that do in terms of how trigger factor, for instance, interacts. That's something we'll talk about in the next module, and we'll be getting there on Wednesday, I hope, if not Friday. So with that, we're going to close discussions of module 1 in the ribosome with looking at some biotechnology and thinking about how we can use this fundamental understanding of the ribosome to do some new things.

And so we're going to talk about re-engineering translation and ways to use this machinery to incorporate unnatural amino acids. And so to begin thinking about this, we can just consider some questions. And so many of us in this room are chemists or chemistry majors. We can think about organic chemistry, so 5.12, 5.13, and all of the different organic transformations that are presented.

So if we think about all these organic transformations and how they're available to synthetic chemists, we see a lot of versatility. And we can simply ask ourselves, can such versatility be achieved for protein modification? What is the toolkit? How can that toolkit be expanded? And then thinking about this further, can we use the translation machinery?

So is it possible to modify the translation machinery to allow us to make peptides or proteins that have unnatural amino acids? So amino acids are moieties that are not the canonical ones. And can we do this in cells? Can we do this in a test tube? And if we can, what does that provide us with in terms of possibilities? So the answer is yes, and we're going to focus on the how and strengths and limitations in terms of our discussions of this machinery here.

I also note-- I believe, JoAnne, this will come up. Will you be talking about this in the nucleotide parts, too?

JOANNE STUBBE: If we get that far.

ELIZABETH NOLAN: If we get that far. So in addition to here, this may come up again towards the end of the course, as a tool. So hopefully we'll get that far, because that's exciting. So let's think about re-engineering translation. And we can think about two things. We can think about the genetic code here, and we can think about the ribosome. And so I'll just present you with the

questions.

If we consider the genetic code, what can be done to this genetic code to change an amino acid in a protein? And if we think about the ribosome, what can be done to the ribosome to change an amino acid in a protein? And effectively, can we expand the genetic code to encode something other than what it's supposed to encode? So can this code allow us to encode an unnatural amino acid?

And from the standpoint of the ribosome, is it possible to design new ribosomes? So can we make a new ribosome that can incorporate unnatural amino acids into proteins? So these are separate but related, and we're going to first discuss basically reassigning-- is it possible to reassign a codon? So why would we want to do this? And let's think about that for a minute.

And what do I mean by expanding the genetic code? So if we think about the genetic code, we all know that it encodes these 20 amino acids building blocks, there's the start codons and the stop codon. And effectively, the codons are all used up, right? There aren't extra codons floating around that we could poach and assign to something else here. So can we overcome this? And why would we want to do that?

Just broadly, if we think about being able to put something other than a natural amino acid in a protein at a specific location-- so exactly where we want it-- that opens up many possibilities for experiments. And we can think about those experiments both happening within a cell or outside of a cell. And these are experiments that just wouldn't be so easy or feasible otherwise.

So maybe we'd like to study protein structure. What could we do? So fluorine is used in NMR quite a bit. Imagine if you could site-specifically label an unnatural amino acid that has a CF3 group, for example, and use that in your NMR studies. So that's something you'll get to think about in the context of problem set two. Ways to study protein function, protein localization. So for instance, instead of attaching GFP, which is big, to a protein of interest, maybe it's possible to incorporate a fluorescent amino acid that lets you see that protein in the cell.

Protein-protein interactions. And maybe we'd like to make a new protein that has some desired characteristic. So there's a lot of possibilities to such technology. Just to keep in mind, what do many of us do? Many of us are familiar with site-directed mutagenesis, where we can change an amino acid in a protein. And we learn many, many things from this, but it is limited

to naturally occurring amino acids. Right? So we'd like something more versatile.

If we think about strategies also just a little bit, backing up here. OK, the first thing I'll just point out is that how I'm going to divide this, just in case this wasn't clear, is considering the native ribosome and then considering engineered ribosomes. And this is where we're going to focus today.

And if we consider strategies, other strategies to incorporate unnatural amino acids, and I guess I'll call these standard, we can imagine chemical and biosynthetic. And I'm not going to go over a plethora of examples for either route. There'll be some slides included in the posted lecture notes that gives examples and pros and cons.

But one example I will give here is just thinking from the standpoint of a chemical modification, what's an example and why we might want to do better. OK, so this is independent of something like site-directed mutagenesis, where you're having an organism do the work. So if we just consider an example of a chemical modification, there's certain amino acid side chains that are amenable to modification.

So imagine you purify a protein, and you want to somehow tag that or label it, right? One option is to modify cysteine residues. And so iodoacetamide and related reagents are commonly employed, so imagine that you have some cysteine. You can react this with iodoacetamide that has some R group, right? What happens?

Here, OK. You can get a covalent modification, and maybe this is a fluorophore or something else, right? So this is terrific, but what are some potential problems?

AUDIENCE: Sorry, would this be a way to modify the amino acid before it's incorporated into the protein? Or would this be something you would do to modify the cysteine in an assembled protein?

ELIZABETH NOLAN: Yeah, this would be after the fact. So imagine you have some protein. You've isolated your protein, and you have some cysteine. Right, and you'd like-- for some reason, you'd like to modify this protein. So maybe a fluorophore to see it. Maybe you know, a CF3 group for NMR here, which then gets to the point, what are possible problems with this?

AUDIENCE: Do you have to use a mild base to be deprotonated, or is it maybe deprotonated based on where it is in the protein?

ELIZABETH Yeah, so that gets to an initial issue, which is what's required to have this chemistry to

NOLAN: happen? Right? The cysteine needs to be deprotonated. So probably the pH of your buffer is going to be elevated some. Does your protein or enzyme like that or not? Maybe, maybe not. Yeah?

AUDIENCE: You can also run into selectivity issues-- I mean, having free cysteine residues isn't common, but it could be a potential problem.

ELIZABETH NOLAN: Yeah, so you need-- well, it will depend on the protein, right? Is the cysteine free or a disulfide? Is it a native cysteine, or have you done site-directed mutagenesis first to put this cysteine in the position you want? Right? And then what happens if your protein has multiple cysteines building on what Rebecca said, and you want to have this label at a site-specific location? Right? What are you going to do about that?

Are you going to have non-specific labeling? Are you going to mutate out the other cysteines? If you do that, what could that mean for your protein fold or function? There's a number of caveats that need to be considered. Nonetheless, it's a possibility to do. In terms of time, this is a pretty extreme example, but I'll just show one example here in thinking about this whole process and what you do, which also builds upon Rebecca's question.

So imagine a protein with two subunits. And subunit 1 has a cysteine, and subunit 2 doesn't. So for some reason, you want to do this labeling. This is actually a protein from my group. And we wanted to stick a fluorophore on it. So we have a cysteine on one of the two subunits. You can run this reaction and get this fluorophore modified form here. And then you can see that's the case, looking at SDS-PAGE.

So here we're looking at Coomassie stain that shows us total protein, and we see there's two subunits, 1 and 2. So the molecular weights are a little different, and we can separate them on this gel. And then if we look in the fluorescence channel, what do we see? We only see fluorescence associated with subunit 1 and not subunit 2, which tells us our labeling strategy has worked well. Like, what we're showing in this equation.

But what's everything that needs to be done? Well, we need to overexpress the protein in some organism. In this case, E. coli. We need to purify the protein. And once we have this purified protein in hand, we need to do the chemical reaction for the labeling. And then we need to purify that product somehow, and that's going to depend on the system you're working at. And then it needs to be analyzed, right? You always want to know what you're working with, right?

So was this reaction to 100%? Did we end up with a mixture? If it's a mixture, what to do about that? So what does this mean in terms of time? And this is not for all cases, OK? This is for this exact case involving this protein shown as a cartoon here. So it takes about six days from start to finish to overexpress and purify it. Steps 2 to 4, based on the purification, we do another four days, right?

So that's 10 days from start to finish, just to get this protein you'd like to use in your experiment. Right? And you can imagine if somehow a label could be put on in vivo, during this initial step here, that that would save some time at the end of the day. So before moving on to what's done for unnatural amino acid incorporation by what we'll call the Schultz method out of Professor Peter Schultz's group, just to think about biosynthetic methods for a minute.

So some common ones are done for structural studies. So for instance, you can imagine feeding an organism something like selenocysteine or selenomethionine. Another example is labeling nitrogens or carbons for NMR, where the organism is fed, say, a labeled amino acid, maybe with N15 or C13 there, right? So that's just a biosynthetic method, where you're changing the growth conditions, rather than doing something to manipulate the genetic code or the ribosome.

So what's the conclusion here? What we want is we want a method of site-specific incorporation of unnatural amino acids in vivo. So in a cell and in a desired organism, depending on what you want to do with high efficiency and also fidelity, so getting back to that idea and before.

OK, so why do we want to do this in vivo? It allows for studies within cells, and you also can purify protein from cells, so you can do in vitro experiments as well. OK, and you can imagine, if you could have all of the pieces of this machinery in a cell, maybe there's some technical advantage to that. So this is what we're going to consider here.

So to this question, can the ribosome incorporate unnatural amino acids into proteins? Effectively, what do we need to think about? One, we need to think about relaxing the substrate specificity of the aminoacyl tRNA synthetase to accommodate some unnatural amino acid, right? Somehow that unnatural amino acid needs to get to the ribosome. So if this can be done, and we can make a tRNA that has an unnatural amino acid attached to it, can this aminoacyl tRNA get to the A-site and do the work?

So this is the method we're going to talk about in some detail for the rest of today and into Wednesday, this Schultz method. So the idea is that there's a tRNA that's dedicated for this unnatural amino acid. We see this unnatural amino acid shown here, where the UAA is indicated by probe. We need an aminoacyl tRNA synthetase that will take this unnatural amino acid and attach it to the three-prime end of the tRNA to give us this aminoacylated tRNA.

And then what? Imagine this tRNA can make its way to the ribosome. What happens? We need a codon for this aminoacyl tRNA. It needs to carry the anticodon, and we're going to talk about this in some more detail in a minute. So we can have a plasmid that has the DNA with the gene of interest in it, right? This plasmid DNA can be transformed into, say, E. coli that has this machinery here.

We can have transcription to give the mRNA that is from this plasmid DNA. And then imagine translations such that this unnatural amino acid is incorporated. So effectively, where we're going is that we need a general method. We want this method to be broadly useful, where we can genetically encode this unnatural amino acid and have it incorporated in response to a unique triplet codon, here.

So in thinking about that, what are the pieces that we need? And we'll think about E. coli for the moment, but this could be other. So yeast, mammalian cells, right? Let your imagination run wild with this here. When the incorporation of the UAA in response to a unique triplet codon.

So if we're going to do this, what do we need? OK, effectively, we need some new components of the trans-- like protein biosynthetic translation machinery, right? So we need to rewind and think about the whole translation process. OK, so the first order of business is that we need a unique codon. Right?

So this only designates or uniquely designates the UAA. And so we need to ask, where does this come from? Because we just went over the fact that the codons are used up for amino acid start and stop. We need a new tRNA. OK, so this tRNA needs to be specific for the unique codon. OK, and we need the corresponding aminoacyl tRNA synthetase, right?

And we need this to load the unnatural amino acid onto the unique tRNA here. So what is a key feature of all of this? A key feature is that if we want to do this in some organism, we need this machinery to be orthogonal to the machinery in that organism.

We cannot have cross-reactivity, because then there's not going to be any selectivity of this incorporation. So no cross-reaction. So what do we need to consider, in terms of these? We need to think about all of the machinery, right? And I just list some considerations here.

So this new tRNA can only allow for translation of the codon for the UAA. It can't be a substrate for any of the endogenous aaRS, because then it will become loaded, potentially, with the wrong amino acid. So think back to lectures 2 and 3. This new aminoacyl tRNA synthetase can only recognize the new tRNA and not endogenous tRNA. So cross-reactivity again.

This unnatural amino acid also can't be a substrate for endogenous enzymes. And also keep in mind, there needs to be some way to get this unnatural amino acid into a cell if we want to do this in a cellular context. So there's just transport issue that needs to be kept in mind. Will this unnatural amino acid get into the cell? OK, so what we're going to do is consider these requirements and what was done to build up this methodology during initial work.

So the first issue is this unique codon, and what is its identity here? And so if we consider the 64 codons, they're used up with the 20 common amino acids. We have the three stop codons and the one start codon. And so in thinking about this, can ask, do we really need three stop codons?

We certainly need our start, and we need codons for the amino acids. But is there some wiggle room here? And so in terms of these stop codons, we have TAA, TAG, and TGA. And these all have names. Ochre, amber, and opal. OK, and so the idea we're going to see is just the question, can we reassign a stop codon?

And can we reassign a stop codon such that it's the codon for the unnatural amino acid? And so basically, if we want to reassign a stop codon, how do we choose? Right? So two things to consider. One, how frequently is each stop codon used? So what do we know about that? And then does this stop codon terminate essential genes? So we can imagine that if we were to reassign a stop codon that's used frequently by E. coli or another host, or if we were to reassign a stop codon that's important for terminating the synthesis of essential genes, in either case, the outcome could be pretty bad, right?

So what was found in thinking about those issues is this amber stop codon, TAG, one, it's the least frequently used. And just for an example, about 9% in E. coli and about 23% in yeast for terminating genes. And additionally, it rarely terminates essential genes. OK, so based on this,

it was decided to reassign TAG as the codon for the unnatural amino acid.

OK, so we've gotten through to here. So the question is now, what about requirements 2 and 3? So yeah?

AUDIENCE: I have a question. So it seems interesting to choose a stop codon to change because if the stop codon messes up, it seems more catastrophic to us all than one of the other redundant amino acids. Like, why use a stop codon? I think it's interesting.

ELIZABETH NOLAN: Yeah, so there is a risk. There's certainly a risk, right? But these considerations were made to try to diminish that risk. Right? So you could make the argument that maybe all of these stop codons aren't essential, right? And what is more deleterious? Will it be to try to use a stop codon that's infrequently used, or to reassign a codon that's for an amino acid that comes up in many, many different proteins in the cellular pool? Right?

So there's a judgment call there. But if we consider in E. coli, this TAG stop is for about 9% of proteins. How does that compare to, say, reassigning one of the codons to incorporate a lysine or a valine. I don't know, but that was just want to think about, how frequently is that codon used? Because certainly there's different codon usage in different organisms. Do you have something to say?

JOANNE STUBBE: So it depends on what you want to put the unnatural amino acid in for. So if you want it in endogenous levels, it could be a problem. But if you're overproducing your protein--

ELIZABETH NOLAN: Yeah, it may not be a problem.

JOANNE STUBBE: Then it's not a problem, because you induce, and then you flood it with that and you get high levels of cooperation. So it depends on what your purpose is.

ELIZABETH NOLAN: Yeah, so is JoAnne's point clear to everyone? So you could imagine expressing at an endogenous level, right? Or you could imagine causing the cell to overexpress the proteins, like off a plasmid, like what many of you have done in lab class or maybe in research there for that. Are there many examples of reassigning a different one?

JOANNE STUBBE: I think it's really tough. I mean, inside the cell, you really do have problems if you don't re-engineer, because you get truncations also.

ELIZABETH NOLAN: Yeah, we're going to talk about that. So there is a big problem about the stop that we're going to talk about, once we get to how we have this done, which is premature termination.

AUDIENCE: I'm kind of confused more or less at that point, because stop codon, we're just using because it's not currently-- it doesn't go for anything, it just ends, like, endogenous sequences that are not the UAA so if we replace it, we might not get those. But we'll get the one that we're trying to synthesize.

ELIZABETH NOLAN: Right, so we need a codon for the unnatural amino acid. And right now, we're limiting our space to triplet codons, which is what was initially done when this type of methodology was developed. So the question is, we have four options in terms of bases.

AUDIENCE: That are not coding, right?

ELIZABETH NOLAN: No, no, in terms of our codons, right? So three, right? Four, three, so there's 64 codons, and they're all used up.

AUDIENCE: Yeah.

ELIZABETH NOLAN: Right, so there's not some extra.

AUDIENCE: Yeah.

ELIZABETH NOLAN: So then what can be reassigned?

AUDIENCE: These stop-- so the stop.

ELIZABETH NOLAN: Well, yeah, well, we can't reassign the start. Then there's no proteins, right? There's only one start codon. So the thinking was, is a stop codon dispensable? Right? And then a decision was made, based on basically the frequency of use of the stop codon and whether or not the stop codon terminates essential genes.

Is this something foolproof? No, there's major problems in terms of yields that come up as a result. And we'll see that on Wednesday, right? But you need a starting point to get a method underway. So where will we begin tomorrow is talking about where this tRNA and the aaRS come from to do this.