Clockwork Genes: Discoveries in Biological Time
Lecture Four—The Mammalian Timekeeper
Michael Rosbash, Ph.D.

1. Start of Lecture Four (00:16)

From the Howard Hughes Medical Institute, the 2000 Holiday Lectures on Science…This year's lectures, "Clockwork Genes: Discoveries in Biological Time," will be given by Dr. Michael Rosbash, Howard Hughes Medical Institute investigator at Brandeis University and Dr. Joseph S. Takahashi, Howard Hughes Medical Institute investigator at Northwestern University. The fourth lecture is titled "The Mammalian Timekeeper." And now, to introduce our program, Howard Hughes Medical Institute's senior program officer, Dr. Dennis Liu.

2. Introduction by HHMI Program Director Dr. Dennis Liu (01:07)

Welcome back for the final lecture in our year 2000 Holiday Lecture series. Dr. Takahashi's presentation is titled "The Mammalian Timekeeper." So far in this series, you've heard about the global forces that have shaped the evolution of circadian rhythms. You've also heard about brilliant work making use of the advanced molecular biology available in fruit flies, and in this next lecture, we'll delve deep into the molecular details of how our own clocks work. This lecture, like the others in our series, features animations developed by a team here at HHMI, and we hope that they're entertaining and illuminating. The animations from this year and previous years' Holiday Lectures are available on a web site that we call BioInteractive.org. If you go to BioInteractive.org, you'll find lots of interactive demonstrations, other animations, and virtual laboratories. Joe Takahashi received his Ph.D. in biology from the University of Oregon, and, in fact, he graduated just a couple of years before I came to Oregon to begin my own graduate studies. At Oregon, Joe worked with Michael Menaker, whose laboratory was devoted to understanding human behavior, and there's really no other way to say it -- Mike Menaker's lab was a zoo. It was full of interesting people, and they worked on every conceivable animal, from slugs to bats and from chickens to wolves, and I'd go often to the Menaker lab, even though I worked in a different lab, for conversation, both scientific and otherwise. I'd ask friends of mine who worked in the lab what they were doing, and they'd say things like, "I'm doing a melatonin assay first developed by Joe Takahashi." "I'm following up on experiments done by Joe Takahashi." "I'm running an experiment on equipment built by Joe Takahashi." And when I asked Mike Menaker about doing a rotation in his lab, he recommended that I read a paper written by Joe Takahashi. "Who was this Joe Takahashi guy?" I wondered. Well, eventually I got to know Joe personally and to talk science with him directly, and it's always a thrill, which you've had a small taste of this morning. So back in Oregon, we knew that Joe would go on to do great things, and here he is, an HHMI investigator and a noted expert in his field. Well, it's time to turn it over to another short video to introduce Joe, and then at the end of our program, HHMI president Tom Cech will return for some closing remarks.

3. Introductory Interview with Dr. Joseph Takahashi (03:37)

It's hard to imagine a more interesting job because I get to do almost anything I want to do. It's one of those wonderful jobs where you have the freedom to just pursue interesting problems, and I think it is important for us to try to pursue problems that are important and important to human health. I think that we, as biologists... need to think about how we can sort of try to educate everyone more broadly about why science is important... how biology impacts our lives. Someone who's interested in business school or law school should really still have some understanding of biology, I think, and the way that our understanding of the human genome in the future is really going to impact society and how we have to live in the future. What do I think that it takes to be a good scientist? I would say one of the most

important ingredients is curiosity. You really have to be just naturally inquisitive and curious about the world around you. Now, that doesn't mean you're going to be a scientist, but I think you have to have this internal sort of drive... to try to understand nature and what is going on in nature. I think that's probably fundamental. Do you have to be a good student? I would say, in a traditional sense, no, you don't, really. They need to be relatively intelligent, somewhat organized, but I think the most important aspect is drive, is they really have to be motivated to understand and answer that question. You really can be many different kinds of people in science because science is so diverse, and so I think that there are many opportunities for people who might think that they're not cut out for science. They just need to try it out and really find some of the opportunities that are really available, you know, to explore it.

4. Review: Animation of mammalian circadian neuroanatomy (06:05)

Hello. It's great to be back. I wanted to thank Dennis Liu and his staff for the incredible job that they've done this year in helping Michael and I out and also Ann Sutherland and her staff in putting together this production, which has really been a learning experience for Michael and I, I can assure you. In this second lecture, I'd like to now turn to the mammalian story again, and if we could begin with that first short animation, I want to take you back to my first lecture, in which we reviewed the idea that we have a clock in our brain and that our eyeballs receive light information. It's carried down to the optic nerve, into the base of the hypothalamus, into these two winglike structures, the suprachiasmatic nuclei, which are composed of thousands of neurons that operate in a network. Each of the neurons fires during the daytime, and indeed in vivo, all the neurons fire in synchrony together to form a circadian output, and as we've heard yesterday and today, the basic mechanism of this cell feedback loop occurs in the cell.

5. Genetic approaches in mice; the model mammalian system (07:26)

So today, what I'd like to do is to really review for us what we have tried to understand in mammals, and I take you back to this slide of Ron Konopka and Seymour Benzer... because throughout my own career, which began in the early eighties -- or late seventies, actually -- we always were interested in trying to understand what molecules compose the biological clock in mammals, but we were frustrated in our efforts to find and get our hands on those molecules, and it was really not until Ron Konopka's discovery of the period gene, or mutant, that we had a new approach to try to understand what these molecules might be composed of, and so following the cloning of the period and the timeless genes in the eighties and nineties, we began to turn our attention to genetic approaches, which were really trailblazed by Seymour Benzer and Ron Konopka, and because we were not able to clone the period gene in mammals, even though it had been cloned in flies in 1984, it was very difficult. It turns out the sequence is quite divergent. What we decided to do was to go back and actually do what Ron Konopka did but this time, to use a mouse instead of a fly, and that was to find mutants in mice that have timing defects, and so, this next slide shows one of my favorite etchings from Barry Moser of the Queen Mouse from "The Wizard of Oz" to illustrate our favorite organism in the laboratory today. About ten years ago, we switched to the mouse as a genetic model system because, arguably, mice and humans, ironically, are the best experimental, or mammalian, model systems for doing genetics.

6. Mutagenesis in mice (09:34)

What we did, shown in the next slide, was to create random mutations in the germ line of mice, and the way you do this is, you treat them with a chemical -- ethyl-nitroso-urea or ENU. You inject male mice. It makes them go sterile, but then they recover fertility a few weeks later. When they do, those germ cells are mutagenized, and you can then cross this mutagenized male to normal female mice to produce a first generation of mice that are carrying gametes that were mutagenized in this mouse here, OK? And so we call these "generation one mice," which are carrying heterozygous mutations randomly throughout

the whole genome. So in our first screens, which were done with my very close colleague Bill Dove at the University of Wisconsin at Madison,

7. Genetic screens to isolate circadian clock mutants (10:41)

we initiated a genetic screen together with Larry Pinto and Fred Turek at Northwestern to search for mice that might have abnormal rhythms, and so here's an activity record, which you've seen many of. This is a record of a mouse in the screen that looks normal. It has a normal pattern of running at night and a shorter period in darkness, and, in fact, this histogram here shows you the period values for the first 300 mice that we screened, and you can see that the average is about 23.7 hours, just like this mouse here, and the distribution is very tight, OK? But a single mouse, mouse number twenty-five -- in fact, it was the twenty-fifth mouse that we screened -- had a very abnormal pattern. It was about one hour longer, on average. As you can see here, it's way out here, six standard deviations away from the mean, and this is the record of mouse number twenty-five. It had a clock that was one hour slower. Now, the important issue, of course, is whether this is really truly a mutation and can it be transmitted to the progeny of this mice, and we do this by test crossing. So we would take this mouse number twenty-five -- luckily, it was a male -- cross it to normal females, and ask, in the progeny of that cross, can we see this mutant phenotype again? And the answer was yes, and indeed, half of the offspring were mutant, and half were normal, which was consistent with the notion that this mouse was carrying a single gene mutation and that it had only one mutant copy of whatever allele that might be, this 50/50 distribution. The other conclusion we can make early on here is that the mutation appears to be dominant, and the reason we say that is, the way we did the screen, only half or only one allele has been mutagenized in all of these mice. So any mutation we detected in this particular screen must be dominant.

8. Genetic analysis of the circadian clock mutant (13:07)

Now when we crossed heterozygotes, mice that look like this, to then try to produce our first homozygous mutant animal, OK? This is actually a record of the first litter that we obtained in such a cross that could have produced a homozygote, and so in such a cross, you would predict a 1 to 2 to 1 mendelian ratio of 1/4 wild type, 2/4 heterozygotes, and 1/4 homozygous mutant, and that is exactly what we got, incredibly. So here is a wild type mouse. These are two heterozygotes, and this is a homozygous mutant. We named the mutation "Clock" based on its phenotype, and, interestingly, the homozygous mutant phenotype is much more extreme than the heterozygous phenotype. That is, the mice have a 28-hour period, which is hard to see here upon release in constant darkness, which then degrades, and they completely lose their circadian rhythm. Because we can see a difference between heterozygotes and homozygotes and because heterozygotes are sort of intermediate between wild type and homozygous mutant, we call this kind of mutation "semi-dominant." We can actually see the phenotypic consequences of the mutant allele, even as a heterozygote, OK?

9. Phenotype of an SCN neuron from a circadian clock mutant (14:46)

Now, in the next slide, we did a very interesting experiment with Erik Herzog and Gene Block at the University of Virginia in which we looked at the single cell phenotype from cultured SCN neurons using this planar microelectrode array technique that I talked about yesterday, and you might recall that using this measurement, we could show that individual SCN cells were themselves competent circadian pacemakers, and so in this experiment, what we've done is to look at cells taken from the three different Clock genotypes of mice and ask, "What is the effect of the Clock mutation at the level of single cells?" And the answer is, as we saw before in wild types, the wild-type rhythm has an average period -- this is the period of the cells in culture -- that is almost identical to the average period of the animal's behavior in vivo -- 23.6 or 23.7. In heterozygotes, the period is one hour longer, and indeed, these individual cell rhythms are, on average, one hour longer. This is incredible to see this correspondence of the phenotype

between a single cell and a whole organism... and indeed, in homozygous mutants, SCN cells actually fail to generate persistent circadian rhythms and are really consistent with the loss of rhythm phenotype that we see at the level of the whole organism. So

10. Impact of the Human Genotype Project on clock research (16:34)

this set of experiments strongly suggested to us that this mutation was very interesting. It's a very extreme phenotype -- 28-hour period and loss of rhythm -- and so, of course, we wanted to understand or to know what the gene was, and back in 1994 when we had fully characterized the Clock mutant, the Human Genome Project had just begun, and we were incredible benefactors of resources from that project because Eric Lander had just produced the first genetic map of the mouse in 1992, and as the resources for that map was generated, we used those markers immediately. It was almost perfect timing,

11. Positional- cloning analysis to map the *Clock* gene (17:26)

and so to actually identify and find the gene, we used a technique that is very common now in trying to identify human disease loci that are caused by single genes, and this method is called "positional cloning." What we do is to map the location of the mutant phenotype using DNA markers, and initially, we could easily find that it was on chromosome 5 of the mouse, and then with higher and higher resolution, we could then zone in or zoom in on where on mouse chromosome 5 the Clock mutant might be located, and what we could say at this point is, it had to be somewhere between here and here, and eventually, we reduced it to these two markers so that we argued genetically that the mutation must be here, somewhere in here, OK? Now, the important next step is to go from a genetic map to a physical map -- that is, physical pieces of DNA -- and in the early and late nineties, there were a few different ways of cloning large segments of DNA, but one of these, which are called YACs -- not that furry animal, but are actually yeast artificial chromosomes -- these were one way of physically cloning very large segments of DNA. In the green are shown a set of YAC clones that overlap this region. And, indeed at this point, the entire lab, because it was such an intense effort, had to get together and work as a team, and many different people took on various aspects of this job, and I think in my lab, it was a very important feature that they were actually quite friendly and social, and, for example -- to give you an example of some of the fun they had in the lab -- they actually made Clock beer out of one of the yeast strains. In fact, this yeast strain right here, yeast number fifty-five... was actually used to make the beer that you find in this bottle here. They also made their own private label, as you can see... and so I saved my bottle for posterity.

12. Using positional cloning and transgenic mice together (20:01)

Now, at the same time, we used a different cloning vector, bacterial artificial chromosomes, or BACs, to cover this region also, and these turned out to be nice resources because the sizes were smaller. They're much easier to work with, and what we did with BACs was to take an alternative approach, and that was to try to rescue the mutation. This was actually first done with the period gene in 1984 and '85 by Michael Rosbash and Jeff Hall in Mike Young's lab to show that a particular segment of DNA contained the period locus, and so we actually used the same strategy with Clock-mutant mice, and what we did was to create transgenic mice that might be carrying these large segments of BAC DNA by microinjection into the pronucleus, and so in these kinds of experiments, you'll get mice that carry and integrate this DNA and then can transmit it to their progeny. After crossing those mice, we could then ask what happens when we put this piece of normal DNA into a mutant mouse? It's really a gene therapy experiment in a mouse, and, incredibly, what we see here is, these are four Clock-mutant mice that have the long period and loss of rhythm phenotype, and these are four littermates that are also Clock-homozygous mutants, but they have this BAC transgene, and their behavior, circadian behavior, is completely normal. There was a complete restoration of function by this piece of DNA,

13. Map of the *Clock* gene region (21:48)

and so this shows you a current map. Turns out, there are about four and a half genes in this region from complete genome sequencing of this region... and these are four BAC clones that we made transgenic mice out of, and it turns out, the yellow clones are the ones that rescued the rhythm -- the orange clones failed to rescue -- and just based on the rescue pattern alone, you could argue or convince yourself that this large gene here must be the Clock gene, and, indeed, it was. This turned out to be an interesting gene. It's about 100,000 base pairs in size. It has twenty-four exons. We joke, one exon for every hour of the day.

14. Structure of the *Clock* protein (CLOCK) (22:40)

And it encodes a very interesting protein. So back in 1997 when we identified the CLOCK Protein, only the period and timeless genes were known in Drosophila, and interestingly, both period, which is shown here, and timeless had no obvious DNA binding motifs on them, and so CLOCK was especially appealing at this time because it had a clear basic helix-loop-helix domain, shown here in the green, and the basic region is known to be a DNA binding domain, as well as the helix-loop-helix and PAS domains, which were found, incredibly, in the period protein, which are protein interaction domains. The final interesting feature of the CLOCK Protein was that the C-terminus was glutamine rich, and this is a signature of the activation domains of transcription factors. So to summarize before I take a question from the house... we used a genetic approach to find a mouse mutant. That mouse mutant then allowed us to identify the underlying gene using the methods of positional cloning and transgenic rescue to identify a very interesting protein -- CLOCK, which is a transcription factor.

15. Q&A: What would you do after understanding the mechanism of the biological clock? (24:06)

So if I could have the first question from the house, let's try the back of the room right there. Yes, you.

Hi. I'm Natasha Wilson from the Potomac School, and I was wondering, once research in circadian clocks is sufficiently over and you've gotten a general knowledge for how circadian clocks work, what are you, as researchers, planning on doing with this information, for instance, like, with gene therapy or anything like that?

Right. So I think listening to Michael and I talk, you might have the impression that a lot of things are known. We have discovered a lot of things, but, clearly, a lot of things are really not known, and so you should keep it clear in your minds that we have sort of identified a set of genes -- I'm going to tell you about the set in mammals -- and so we sort of have a set of players, but we have very little understanding of how they actually function, how we get twenty-four hours out of the segment, and so we're making hypotheses to test, but I think, just on the Clock problem alone, we still have a long way to go in understanding mechanisms. I think the other interesting feature is, do these apply to human conditions? And so at the end of my talk, I'm going to give you an example of that. Finally, I think I'm interested in actually doing some new things. I'm going to use genetics to actually find genes that control other behaviors, like learning and memory and more complicated functions. So the

16. Student Question: Can you phase-shift a mammal with blue light? (25:47)

next question is from Miami.

And my question was, if you can phase a fly with a blue light, can you also phase a mammal?

So the answer is, yes. In mammals, blue-green light is actually the most effective light for phase shifting our clocks. The only qualification I need to make with that is, in mammals, we don't really know what the photo pigment is, and, as I'll tell you in the second half of my lecture, Cryptochrome is unlikely to be that photo receptor, as we saw in flies.

17. Q&A: Have you done overexpression experiments with *Clock*? (26:24)

Next question is from East Lyme. Go ahead, East Lyme.

Hi. My name is Anja Deshmukh, and I'm a sophomore at East Lyme High School. I was wondering, have you done any overexpression experiments with Clock?

A good question, and indeed, we have. In those mice that overexpress the Clock locus to be used for rescue, if you express that Clock gene on a wild-type background, what we find is that the mice have a shorter period. They get shorter by an hour, in some extreme cases, two hours.

18. Q&A: What experiments do you use to see the difference between mutant and wild-type genes? (27:05)

Next question is from Moscow. Go ahead, Moscow.

This is Ironica, Lyceum Number 13. I'd like to ask a question. What kind of experiments were used to understand interaction of the mutant genes and live genes?

So what kind of experiments do we use to see the difference between mutant genes and the wild-type genes? And so in the case of the Clock mutation, the difference was caused by just a single base pair difference, an A-to-T transversion. That was it -- one change in the DNA. That turned out to be in an interesting place in the Clock gene. It was in the 5 prime splice donor site of an exon in the protein, and that mutation caused that particular exon to be skipped, thus producing a deletion in the middle of the CLOCK Protein, and so it was really DNA-based.

19. Q&A: What other kinds of mutations are there in the *Clock* gene? (28:08)

FThe next question we'll take from the house. Right here.

You said that most -- that the Clock mutation was a deletion. What type are most other mutations in other genes that control the biological clock?

So most of the mutants that were made or have been made in flies and mammals have been made with chemical mutagens that induce point mutation, just single base pair changes, and so, many of the mutations are actually single amino acid residue mutations, but as you saw for the period mutation, that nucleotide actually caused a stop codon in the case of the Per0, and so you can get stop codons, or in the case of the Clock mutation, it causes splicing mutation. So we get a wide variety.

I'm afraid we're out of time for questions, and so let me continue on with the next slide.

20. Function of CLOCK (29:06)

So as you already heard from the Drosophila story, the function of the CLOCK Protein is really to act as a positive acting transcription factor in concert with its partner, which we call "BMAL" in mammals. BMAL is the homolog of the Drosophila cycle protein, and they are both basic helix-loop-helix PAS

proteins. They form a heterodimer, and they bind to an E-box motif found in the control region of Per genes, and, indeed, we discovered the BMAL protein in a collaboration with Chuck White at Harvard in which we used an interaction screen using CLOCK in yeast to see what proteins CLOCK would bind to, and BMAL was one of those proteins. When we then looked at the mammalian version of the period genes, which had been cloned by two other laboratories in the world -- Hajime Tei in Tokyo and Cheng Chi Lee at Baylor -- we actually found that the upstream control regions of the mammalian Per genes contained E-box motifs, and so, through biochemical experiments, we could provide evidence for this kind of model that CLOCK and BMAL activate the transcription of the Period 1 gene, and that, indeed, in the CLOCK mutant protein, the protein could bind or interact with BMAL. It could still bind the E-box but was somehow defective in activating transcription.

21. Nine different proteins related to circadian clock genes (30:48)

So in the last three years or so, through both molecular biology and genomics techniques, we now have a set of nine different proteins that are either Clock genes or putative Clock genes, and so we have the CLOCK Protein here and its partner BMAL1, which are positive-acting transcription factors, and then, interestingly, in mammals, there are three orthologs of the period protein, which we call Per1, Per2, and Per3. They all share this PAS domain. There is also a protein that's similar to the fly timeless gene, but recent experiments suggest that this particular version of timeless may be different from fly protein. And finally, there are two Cryptochrome proteins in mammals similar in sequence to the Cryptochrome proteins in flies. However, as I'm going to show you in a minute, their roles appear to be completely different in mammals. The ninth protein is casein kinase 1 epsilon, which is similar to the doubletime mutation that Mike Rosbash talked about, and I'll also be telling you a little bit about casein kinase 1 epsilon.

22. *Cryptochrome 1* and *2* genes (32:13)

Now, Cryptochrome 1 and 2 was really studied by using knockouts in mice. That is a method in which we selectively create a null mutation in these genes, and in experiments done in collaboration with Aziz Sancar and Todo in Japan, we found that Cryptochrome mutations did indeed change circadian rhythms in mice. Knocking out the Cryptochrome 1 gene caused the period to shorten by about one hour compared to wild type. On the other hand, knocking out the Cryptochrome 2 gene caused the period to lengthen about one hour as compared to wild type. But the real surprise came when we made the double mutation of Cryptochrome 1 and 2. In these mice, the rhythm in constant darkness was completely eliminated. These mice have no circadian rhythm whatsoever, and so this suggests that Cryptochrome might be playing a slightly different role in mammals than it is in flies or in plants, where it's a photoreceptor, and, indeed, in experiments that I'm not going to have time to show you, it turns out Cryptochrome is actually part of the negative feedback loop. And so I'd like to go to the first animation...

23. Animation: Role of Period (Per) and Cryptochrome (Cry) genes (33:38)

to illustrate the role of the Pers and Crys. And so what we have here are BMAL and CLOCK, which are the positive activators of actually a set of at least 5 genes. All 3 Per genes and both Cryptochrome genes appear to be under the regulation of CLOCK, based on genetic experiments, and these are activators of all of these proteins. To simplify the animation, we've reduced it to just period and Cryptochrome, and as we saw in Drosophila, what happens is, once the period and Cryptochrome genes are turned on, their RNAs accumulate, proteins are made, but in this case, a number of different dimer combinations can be formed: Per-Per dimers, Cryptochrome-Per dimers, and different combinations of the various Per and Cryptochrome proteins. They then translocate into the nucleus, where they interact directly with CLOCK and BMAL to then turn off the period and Cryptochrome genes. Then as time progresses, these

negative factors, Per and Cryptochrome, turn over and disappear, are degraded, and then the inhibition is relieved, the activation begins, and the start of the cycle begins again.

24. *tau* mutant in the golden hamster (35:08)

Now if I could go back to the slides, the last gene came recently this year as a result of experiments done on the golden hamster shown here... and so I remind you, in the golden hamster, there was a spontaneous mutation, isolated by Martin Ralph and Menaker in 1988, and in this hamster, the period is shorter than twenty-four hours. In homozygous tau mutants, as the mutation is called, the clock is four hours shorter each day. It has a twenty-hour clock. In heterozygotes, the period is intermediate -- it's a 22-hour clock -- and in wild types, it's about exactly twenty-four. So tau, again, is a semi-dominant autosomal mutation that shortens free-running period in mammals and, indeed, was actually the first single gene mutation to be found in mammals back in 1988.

25. Identifying the gene involved in the *tau* mutant (36:14)

Now, in order to find tau, we also had to use a genetic positional cloning approach, but it turned out, it was much more difficult to clone a gene in the hamster because the hamster is not supported by the Human Genome Project, and so all the resources that we had in the mouse were not available in the hamster, and so we actually used some molecular methods to find the gene, and what we did was to actually subtract the DNA from mutants and wild types and clone the differences in the DNAs using a technique called representational difference analysis. This led to a set of DNA clones shown here, RDA-650 and RDA-750, which we eventually were able to use to find segments of DNA that were on mouse chromosome 15. This gave us our first clue, OK? Once we got to this region, because of the genetic map in the mouse, we could find a set of genes in the mouse. We then cloned them in the hamster, found polymorphic markers, and were able to show that they were indeed linked to the tau mutation, and so what we actually found is that there was an interval of DNA about 15 centimorgans in a hamster and mouse that were virtually identical. They had the same set of genes. We call this "synteny" or "conserved synteny." Then using the same strategy, we looked in the human genome, and it turns out, three segments of the human genome are conserved with mouse 15 -- chromosome 8, 12, and 22 -- and in 22, shown here, this gene, casein kinase 1 epsilon, popped out, which became an obvious candidate because the doubletime mutation had just been identified as casein kinase 1 epsilon in Drosophila. We then went all the way back to hamsters, cloned this gene, and found that it was indistinguishable from the tau mutation, OK?

26. Biochemical basis of *tau* mutation on *Casein kinase 1, epsilon* gene(38:26)

Now, what was that mutation in this enzyme? This is a structure, a representation of the structure of this enzyme based on the yeast version of this enzyme. It turned out that the tau mutation occurs in an amino acid, residue 183, that's involved in binding of a phosphate substrate... in this enzyme that puts additional phosphates onto the protein. And so this is a surface view of the same enzyme, and what we found through biochemical experiments is that this single amino acid change in casein kinase 1 epsilon caused an eightfold reduction in the maximal velocity, or turnover, of this enzyme without changing too many other properties, OK?

27. Animation: Role of Casein kinase gene (39:24)

So in the next animation... we're just going to add casein kinase 1 here as a new member of this Clock gene family... and as we saw before, we have CLOCK and BMAL regulating Per and Cryptochrome and casein kinase 1. We'll start with its role in the cytoplasm, which we believe is very similar to what has been found in Drosophila, and that is to phosphorylate the Per protein and make it less stable so that it

degrades, unless the Per forms a dimer with either itself or Cryptochrome. Another role that's been identified in mammals is that casein kinase 1 epsilon may also be involved in either cellular retention or translocation into the nucleus. It's a second role. A third role is that casein kinase is also involved in the turnover of the inhibitory complex in the nucleus as Michael Rosbash illustrated. Once those inhibitory molecules degrade, the cycle starts over, and we see the beginning of a new cycle. Now, if I could go back to the next slide...

28. The effect of *tau* mutation on the *Period* gene's expression level (40:49)

How do we think that the tau mutation actually shortens the period by four hours, OK? So if we go back to the hamster and look in the SCN and use the Per gene as a marker of a molecular rhythm, this is what the Per gene RNA profile looks like in a normal hamster, wild type, shown in blue... and in homozygous mutant hamsters, this is what the period gene profile looks like... shown in yellow. The major difference that we see is that the decline of the Per RNA occurs about four hours earlier in tau mutants than it does in wild type. The decline of the Per RNA is a very interesting part of the cycle because that is the marker for the negative feedback effects of the inhibitory complex, and so this suggested very strongly to us that perhaps what's going on is that the negative feedback is occurring earlier in tau mutants as compared to wild type, and this effect is primarily cytoplasmic. If we could run the next animation...

29. Animation: *tau* mutation causes early buildup of negative feedback proteins (42:04)

this shows you a wild type, as compared to a mutant hamster in this case, and what we see is, the Per genes are produced, but because the mutant enzyme is less effective in phosphorylating Per, we would suggest that Per can accumulate faster in the cytoplasm, then translocate into the nucleus earlier, which leads to an earlier shut-off. The negative feedback occurs earlier as compared to wild type. Now this is in contrast to the actual example that Michael showed, where the casein kinase effect was primarily in the nucleus. So we think the tau mutation is working primarily in the cytoplasmic location, and we're getting opposite effects of period.

30. Summary and comparison of mammalian and fly clocks (42:51)

So if I could go to the next slide... What I've really tried to do is to give you an outline of the molecular components of the circadian system in mammals, and so what I've done is to take the two models that we've been seeing over the last two days for mammals and flies, and what I'd like to do is just to give you a couple comparisons between the systems. Obviously, the two systems are incredibly well-conserved. That is, we see the same set of genes playing a role in both flies and mammals, and, indeed, it was this conservation that allowed us to identify the genes, either in a mouse or in a fly, to find the same set in both organisms. So that was incredibly useful. Now, perhaps the most well-conserved elements are the activators -- cycle and CLOCK in flies, BMAL and CLOCK in mammals -- and the role of casein kinase 1 epsilon. Those are incredibly similar. The negative feedback elements show a lot of diversity. So in mammals, there are more of them -- three Pers and two Cryptochromes and perhaps more -- and also, there's a very important role for the timeless protein in flies, which does not appear to play a role in mammals. The other missing element in mammals is the Cryptochrome timeless light degradation pathway. This does not exist in mammals, and indeed, the way that light gets into the system is through a different set of genes. In the case of mammals, the light input comes in as a positive signal to the Per1 and Per2 genes, and this occurs only in the suprachiasmatic nucleus. So light exposure to a rodent causes a rapid increase in Per1 and Per2 RNA, and so we believe that this is really the site at which light or entrainment information enters this molecular feedback loop in mammals, and this is in contrast to the system in flies, where Cryptochrome timeless appears to be involved. So I'd like to now go on to a short video which introduces the final topic of my talk today, and that is, what role might these genes have in humans.

31. Video: Interview on how *per* and other genes relate to humans (45:29)

I think a really important aspect is how the human Clock is related to the other mammalian and the fly Clock because the Per gene is -- There is actually three copies of it in humans. So evolution, for some reason, has chosen the Per gene to utilize in the higher organisms, and, of course, Per is also found in the mice and the hamsters. So the question is, how do the human Clocks then differ from the other Clocks? And then we can start to address human disorders.

32. A human sleep disorder related to circadian clock (46:14)

So indeed, as we've seen, mammalian Clocks are very similar to fly Clocks... but what about humans as a particular example of a mammal? And so it turns out, one of our colleagues in the HHMI, Louis Ptacek at the University of Utah and his associate Chris Jones, identified a very interesting family that expresses a sleep disorder that's called "familial advanced sleep-phase syndrome." Now what does advanced sleep-phase syndrome mean? It turns out, in these individuals, they wake up about three to four hours earlier and go to sleep about three or four hours earlier on average than normal individuals. That is, they actually -- Some of them, wake up around 3:00 A.M. in the morning and have great difficulty in staying up past 9:00 P.M. and sometimes have a tendency to fall asleep earlier than that, and so this is one family that's been identified by Chris Jones and Louis Ptacek. At Northwestern, my colleague Phyllis Zee, who's the director of the sleep clinic, has found another family that has the same phenotype advanced sleep-phase syndrome, and in that family, it also appears to be inherited. So the next slide shows you the pedigree from Louis Ptacek's family here -- this is a sixth-generation pedigree -- and in the yellow are shown individuals who have advanced sleep-phase syndrome, and in blue are shown individuals who are negative, or normal. The grays show people who have unknown phenotype or who were not tested, but just from this pattern alone, we can conclude that this appears to be a genetic disorder and that it appears to be transmitted in an autosomal dominant fashion, and indeed, Louis and his colleagues have been working very hard in trying to map this location, and my understanding is that they are very close to a solution for identifying this particular gene. So, in conclusion, even in humans, we see evidence that circadian clock genes -- I shouldn't say circadian clock genes, but there are circadian timing defects that appear to be controlled by single genes, and it will be interesting to see whether this particular ASPS pedigree is going to unveil one of the known Clock genes that we already have or whether it's going to be something completely novel.

33. Summary of the four lectures (49:18)

So I'd like to move on with the last video piece... to close out my lecture. As all of you have really seen over the last two days, life on earth has adapted to our cyclic environment by the evolution of cellular circadian clock systems. These systems have shown incredible conservation at the genetic level, and, in particular, the basis of this clock appears to be a feedback loop, a negative feedback loop at the level of transcription of a special set of genes -- these genes are identical in all animals -- but, indeed, when we look more broadly in all living organisms that have rhythms, the feedback loop is conserved. So in Neurospora and in cyanobacteria, we see a negative feedback loop... and this, we believe, is really the basis of the circadian clock system. So if I could have the last slide, I'd like to close by thanking the members of my lab, who really contributed to all the work that I've really been telling you about today. In cloning and isolating the Clock gene, it really took a team effort, and many members of my lab contributed to this effort. Martha found the mouse. David King cloned the Clock gene. Marina Antoch rescued the mutation, and Phil Lowrey here identified the tau mutation, and so I'd like to close by really saying that science is really more than just an individual effort -- and modern science is really a team effort, it's a social activity -- and that I hope Michael and my lecture have illustrated to you the interest and fun of science. Thank you very much.

So, we'd like to start --

34. Q&A: How do Cryptochrome double mutants behave in light/dark cycles? (51:39)

We'll start with a house question first. Let's see. In the gray.

You said that when you were testing levels that had the Cryptochrome 1 and 2 removed in a low-light situation, they didn't have any pattern at all. When they are placed in a place with a light cycle, are they affected by it in any way?

Yes. That's a very good question. So when those Cryptochrome 1-2 mutant mice are placed on light-dark cycles, they have primarily dark activity, but the activity does not appear to be reflecting an underlying oscillator. It appears to be driven by the darkness. So when they're in dark, they're active, and when they're in light, they're inhibited, and you can sort of show that by giving them all sorts of strange light cycles. So if you turn on and off the lights, you can essentially control whether they're active or not, and so the conclusion in those particular mice is that light is just directly influencing activity.

35. Q&A: Does temperature affect the circadian rhythm in mammals? (52:48)

The last question -- I'm sorry -- we'll have to take from Fox Chase Cancer Center.

Hello. My name is Christian Vudas. I'm an eighth-grade student at Baldi Middle School. Does the temperature affect the cycle in mammals?

Yes. That's a very good question. So even in mammals, the circadian clock is temperature compensated, and that's been done by isolating the SCN in culture and changing the temperature. The period remains the same. So I'd like to thank all of you for listening. It's been a real pleasure.

36. Closing remarks by HHMI President Dr. Thomas Cech (53:26)

Thank you, Joe, for another excellent lecture and to the students for the questions. I know from personal experience what a tough job it can be to explain these technical concepts in nontechnical terms to even a very bright audience such as this, and I think that both Michael and Joe have done a remarkable job, and let's have one more round of applause for both of our speakers. Both of our speakers have also reminded us about how many questions remain unanswered for future work. As Joe just explained, we need to learn a lot about the biochemical pathways, the interactions between some of these proteins, the signaling mechanisms, and much more. The same is true for a lot of other fields of biomedical research, and that's where all of you come in because there's really opportunities in the future in the scientific community for bright young people such as you to take on this sort of research, and I hope that these lectures have inspired at least some of you to consider this sort of a career, not necessarily with biological clocks, but there are just a vast number of other subfields of biomedical research where there are equally exciting questions remaining to be answered, and I can't think of anything that's really more important or exciting as we begin this next century. Well, maybe I can think of one thing that's equally exciting, at least in the short term, and that's next year's Holiday Lectures. Our topic in 2001 is something that I know you'll find interesting -- sex. Our speakers will be Barbara Meyer, an HHMI investigator at the University of California at Berkeley, and David Page, who is also a Hughes investigator at the Massachusetts Institute of Technology. That's M.I.T., the Whitehead Institute. Barbara and David are pioneers in the study of what causes an organism to become male or female. Their lectures will explore the biological origins of sexual differentiation and examine possible new treatments for infertility and other medical problems. Since this is our final lecture for this year, I want

to take a moment to thank our staff, the television crew, the teacher advisory committee, the web site developers, and everyone else who worked behind the scenes. Most of all, thanks to all of you who have joined us for these lectures. Thank you, Miami. Thank you, Fox Chase. Thank you, East Lyme. Thank you, Moscow. If you've been watching on television or on the web, please let us know what you think about these lectures. We're interested in both positive and negative feedback because, of course, we can revise things for next year, and if you see our mailing address -- it's going to be listed at the end of the broadcast -- and that's the place where you can send your comments. Finally, I want to thank all of you once more for joining us. You've been a wonderful audience. And now, from all of us here at the Howard Hughes Medical Institute, I want to wish you happy holidays and a great new year.