Seminar 11-4-74

I. Ron:

A. Discussion about the Adenine marker

1. Always check competent cells before using. If cells have been frozen thaw them & use immediately for transformation.

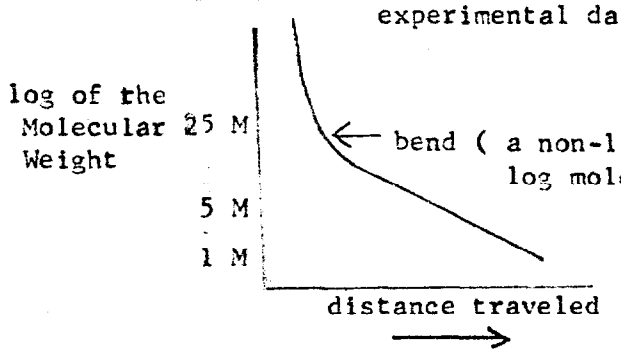
2. Four markers have already been mapped:

Tryp-Tyr	biological	activity	appears	at	2.2-	cm	from	the origin
Histidine	"	"	"	"	3.8	cm	"	" " }
Methionine	"	"	"	"	3.1	cm	"	" " } with T7
Uracil	"	"	"	"	4.5	cm	"	" " } marker at

3. Now the marker Adenine has been mapped; it seems to survive ~~at a lower~~ 3.2. rate. One experiment gave 0.5% survival & another 4-5% survival of the marker after R_I digestion.

4. A careful mapping for the adenine marker was done in the heavy region & it was not found. The entire gel was cut up into 5 mm slices. The adenine marker was located several cm beyond the other four markers, 6.5-7 cm from the origin in the lighter region of the material, when methionine is at 3 - 3.5 cm.

5. It was suggested that instead of expressing the distance traveled by the markers from the origin, description should be made in terms of M.W. (by use of a phage DNA of known M.W. as a marker & comparing its distance from the origin with that of other markers). To do this there must be a linear relationship between the M.W. & the distance traveled by the marker. The heavier the marker, the less distance it would travel.



The bend occurs somewhere between 25 and 5 Million M.W.
The range of R_I digested DNA is ~~5 - 25 Million M.W.~~ about 1 - 15 million.
The heaviest bands from R_I digested DNA may not be in the linear range.
This may indicate that Run the electrophoresis for different lengths of times & compare the distances traveled over the different periods of time by two light bands (e.g., SV 40 as one), which are probably in the linear range, with the heavy band. See if there is a single linear relationship for the movement of the three bands.

6. There is a retardation effect on large molecules (a function of weight), but this can be controlled by changing the % agarose. By diluting the agarose (using a lower conc.), the larger molecules may move more freely.

7. Use the electron microscope for statistically good information on size.

B. Transformation Efficiency

1. With the His_1 marker, there is a difference in transformation efficiency according to dilution. There is no linear relationship in the number of transformed colonies between 10^0 and 10^{-1} plates. The 10^0 plates have more transformed colonies than should occur. All the other markers show linear relationships between the number of transformed colonies & the dilution used. Perhaps in the case of His_1 there is an oversaturation with DNA; by using more cells, more DNA can be soaked up & more transformants obtained.

2. Nontransformed cells may be helping transformed cells in the case of the His_1 marker. Test for this in a reconstruction experiment. Combine cells + DNA for a half hour transformation. Then add DNAase. Make dilutions of the transformed cells. Add untransformed cells to the diluted transformed cells to see if there is an increase in the transforming activity = helper effect. Trance amounts of His may be added to the medium - just enough to give the cells a head start.

3. Reversion is not a problem with histidine.

C. Transforming activity of markers that survive R_I treatment decreases.

1. The reason why this occurs is not known.
   a. This phenomenon may be connected to size change.
   b. It may be due to loss of specific sequences on DNA that are needed for recombination.
   c. New kinds of terminals may be made.
   d. With this R_I treated DNA there may be a different kind of transformation - Ectopic Insertion.

2. Use DNA sheared by passage through a needle. Treat with R_I. Shear first - test for biological activity. Then R_I treat & test for biological activity. Compare the two. Is there any correlation between the survival of the marker with the starting size of DNA? If yes, the phenomenon may be primarily because of decrease in size. If one shears to a size about the same as R_I pieces, then checks transformation for Tryp-Tyr, it will be low but measurable. R_I treatment of this DNA should not decrease the Tryp-Tyr frequency of transformation, because the size will be relatively unchanged. Shear to 20 million M.W. Shear to 10 million M.W. Then in each case treat with R_I. The average M.W. change of the Tryp-Tyro piece will be greater in the 20 million case.

3. What does exonuclease do to activity after R_I treatment? Exonuclease changes the ends by breaking DNA down from the ends. If R_I- generated ends are causing the low transformation efficiency, then exonuclease I brief treatment may restore efficiency. There are Rec^- mutants in B.s.. Contact Don Glazer at Berkely for these specific strains which lack exonucleases.

4. Effect of shearing:

   a. Starting DNA material is 80 million M.W.
   Sheared material is 40 million M.W.
   R_I fragment that contains Tryp marker is about 20 million M.W.
   R_I fragment that contains Aro_2-Tyro marker is less than 13 million.

b. R_I treatment only:
Survival, compared to unsheared parent, is 37% for Tryp marker
26% for His marker

c. Shearing first, then R_I digestion:
Survival, compared to the sheared parent (whose activity was probably 1/2 that of the unsheared parent), , is 57% for Tryp and 71% for His.

d. R_I digestion first & then shearing: Transformation activity should be reduced, if the shearing is done with a very fine needle.

e. Shearing does not operate randomly but tends to break the DNA in half each time.

f. Shear to 10 million (half of what is expected from R_I treatment). Then test the decrease of transforming activity of Tryp-Tyro, before & after subsequent R_I digestion.
Or shear to 20 million & do size fractionation, discarding the smaller pieces, and then test the decrease of transforming activity of Tryp-Tyro, before & after subsequent R_I digestion.

g. Shearing events are not independent of one another in a large molecule. The location of the shear cuts relative to the R_I cuts should be random.

h. Independent of the location of the marker, the size of the DNA introduces another efficiency factor. If the size is gotten down far enough by shearing, then R_I treatment (on the assumption that R_I acts only on certain specific sites) should have relatively less effect on transformation.

i. Compare the activity of fragments of 10 million obtained by shearing & by R_I treatment. These fragments can be fractionated by sucrose gradient or electrophoresis, depending on the distance traveled by the individual fragments. The survival of single markers is approximately the same, except for certain markers such as Lysine, Glycine, and Aro_2. There is less than 10% survival. A change in weight (size) affects biological activity.

5. End Effects:

a. Methionine marker survives 3 times as much as Tryp. This may be due to end effects.

b. Use of R_I - certain markers are always at end or always far away from end.

c. Use of shearing - the markers' position is random.

d. Markers far from an end survive better.

e. Different markers behave differently. The time of integration may be different for different markers.

6. Geometry - location of marker on the chromosome.

a. Though there is experimental evidence that biological activity decrease is due to decrease in the size of DNA, size is not all, we believe.

b. If one treats DNA with R_I, electrophoreses it on gel, & assays the gel for single markers, it is suggested that geometry differences could account for the difference in survival.

i. Survival of Tyr alone is twice as high as Tryp-Tyr together.

ii. Survival of Tryp alone is 7-8 times as high as Tryp-Tyr together.

iii. The lowest survival is for Tryp-Tyr.

iv. His_2 has the highest survival (20 X); it is the most centrally located marker.

c. This suggests a very specific geometry. Biological activity is related to the marker's position on the chromosome.

d. Sheared DNA shows no such systematic effect.

e. R_I treatment gives a definite location of a marker.

II. Dusko: Densitometry

1. To record the band pattern obtained from gel electrophoresis.
2. The scan is very reproducible.
3. SV40 and T7 markers were introduced for positions, to help calculate M.W.s , and to evaluate the noise level; each gives the band width for a homogeneous DNA - a single narrow peak each.
4. To lose the very high noise signals, gel must be removed from quartz tubes. The noise is noise of film & not of instrument. Take the average of many runs & noise signals will cancel out.
5. How much DNA is present between bands is another problem.
6. Do a standard curve using known DNA concentrations as control.
7. In the region between T7 - SV40, about 20 resolvable bands are seen. The relative intensity of the bands is not seen too well:
   a. because the base line is too high.
   b. maybe there are changes in the dye contrast; perhaps dye is washed out at one end of gel.
   c. maybe there are changes in the light concentration; UV intensity may not be uniform from one end to the other.
8. What is shown is that the bands do exist and that they are reproducible.
9. Troughs are places with fewer species of DNA; peaks are sites with a greater number of species of DNA.
10. Histidine is in a trough - in the biggest interband.
11. Factors in band width:
    a. diffusion of the DNA - there is a relationship between M.W. & diffusion coefficient.
    b. optical setup which causes scattering
    c. artifacts
    d. band width as a function of M.W.- diffusion is dependent on M.W. & artifacts are not.

III. Hela:

A. Beneckea Doubling Time

In minimum medium + 2% NaCl + 0.25% glucose: 48 minutes

In complete medium of TB + 2% NaCl 30-35 minutes

B. Crossing Experiments:

Parents: B.s. SB 863 Lys+ Tryp- Tyr- Aro_2- leu- cys- His_1- $Strep^R$

B.s. SD8 Lys- Tryp+ Tyr+ Aro_2+ leu+ cys- His_1+ $Strep^S$

1. Plates with minimal medium + required amino acids

a. SD8 alone (10^0, 10^{-1}, 10^{-2}, 10^{-3} concentrations)
without cysteine - no colonies
with cysteine- no colonies

b. 863 alone, with or without cysteine - 0 colonies

c. 863 + SD8 without cysteine- 0 colonies

d. 863 + SD8 + cysteine

10^0 combined concentration of both kinds of cells - heavy overgrowth of cells

10^{-1} " " " " " " " - still " " " ",
but some individual colonies.

10^{-2} " " " " " " " - individual colonies

10^{-3} " " " " " " " - 143 " " (not as many)

e. Minimum medium + leu + his: parents - crossing -

" " + leu+his+cys: " - " +

When the two kinds of cells were grown together in nutrient broth, when plated, a positive result was obtained - colonies grew on plates with cysteine. Possibly this represents:

i. a recombination of Tryp+ Tyr+ Aro_2+ of SD8 with Tryp-Tyr-Aro_2- of 863 or Lys+ of 863 with Lys- of SD8.

ii. a mutual transformation.

2. Suggestions:

a. The bacteria were grown together in broth with shaking for 48 hours because B.s. lyses without such aeration. But shaking is detrimental to conjugation. Therefore, grow each parent separately with shaking & then do the conjugation for a limited period of time in fresh medium without or with gently shaking.

b. Repeat the experiment with a shorter period of interaction between the cells.

c. See which markers may be segregated (look at many cells using replica plating). Purify the colonies and check out their phenotypes.

d. The parents may be releasing DNA while growing (in broth or/& agar) & the DNA may be performing transformation. Therefore, add DNAase.

3. Testing of Single Markers:

The two kinds of bacteria were grown in nutrient broth with shaking for 48 hours. They were then plated together for 12 days on plates with amino acids minus Tryp, Tyro, Leuj, Lys. The four bacterial colonies that grew from this first crossing experiment of 863 X SD8 were tested for single markers (the colonies were unpurified). **

Colony#	all essential AAs but minus Lys	all essential AAs but minus Phe	all essential AAs but minus Tryp	all essential AAs but minus His	Minimal +His,Tyr, Tryp,Lys, Shikemic Acid, Leu but minus Cys	nutrient agar + 400%/ml Strep.
1	-	+	+	+	-	-
2	-	+	+	+	-	-
3	+	+	+	+	-	+
4	+	+	+	+	-	+

\+ indicates growth of bacteria

\- indicates bacteria doesn't grow

1 and 2 are like parent SD8

3 and 4 indicate recombination (?)

* If all essential amino acids minus cysteine are used, alot of reversion occurs (cell growth). Consequently, in testing for the cysteine marker, minimal medium + only the amino acids required by 863 and SD8 areused, minus cysteine.

B.C.