MAMMALIAN CELL GENETICS

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In our second year at Cold Spring Harbor, we have continued the explorations of gene expression in cultured mammalian cells made possible by techniques of gene cloning and gene transfer, have consolidated insights and developments of the previous year, and have opened up new areas for experimentation. We have been joined by Mitchell Goldfarb, a postdoctoral fellow from R. Weinberg's lab at MIT; Jesse Kwoh, formerly with the Molecular Genetics section at Cold Spring Harbor; and Kenji Shimizu, a microbial geneticist and biochemist from the University of Kyushu, Japan. In addition, Manuel Perucho has been given a staff appointment.

The Physical State of Exogenous DNA in Transformed Cells

M. Perucho, M. Wigler

The genetic content of cultured mammalian cells may be altered—a process classically referred to as transformation—by exposure of cells to DNA as a calcium phosphate precipitate. Transformants are most readily identified by their acquisition of a new phenotype. Previously, we established that under certain conditions cells will stably incorporate up to 4000 kb of exogenous DNA. An extensive series of experiments demonstrating the genetic linkage of acquired phenotypes led us to postulate that transformed cells assemble a structure of up to 4000 kb comprised primarily, if not exclusively, of foreign DNA and that most, if not all, incorporated DNA persists in transformants in one such structure, which we have called a "pekelasome." This hypothesis was confirmed by hybridization analysis of transforming elements after their molecular cloning in prokaryote hosts. Thus, foreign DNA incorporated by mammalian cells undergoes a relatively efficient intermolecular ligation, a process as yet without parallel in microorganisms. We do not yet know whether this process is random or whether it favors certain sequences or even certain molecular configurations. Our initial studies, performed with a Tk^- (thymidine-kinase-deficient) murine cell, LTk^-, have now been further confirmed in that cell and also extensively confirmed in a Tk^- rat cell line, Rat-2, derived by Bill Topp (Tumor Virus section). Other workers have also confirmed our initial observations and have, in addition, demonstrated that foreign DNA is usually integrated into a host chromosome as a single unit. This organization of foreign DNA in transformants may be an important factor to consider in the analysis of expression of newly acquired genes. Moreover, the phenotypic linkage between previously unlinked, newly acquired markers provides a very strong criterion for true gene-transfer events, a point to which we shall return when discussing the isolation of tumor genes.

The Expression of Genes in Transformed Cells

M. Perucho, M. Wigler

Previously, we studied the expression of the simian virus 40 (SV40) early region when cotransferred into LTk^- cells using *tk* as the selectable marker. Following expression of the early region by indirect immunofluorescent staining for T antigen, we (in collaboration with D. Lane, D. Hanahan, and M. Botchan) established that cloned populations of cells expressed T antigen in a heterogeneous pattern (Hanahan et al., *Cell 21:* 127 [1980]). Only a portion of the population were found expressing at any one time, and yet it was impossible to clone out a pure nonexpressing subpopulation. We concluded that expression of the early region was switching off and on during the progression of individual cell lineages. The molecular basis for this switching is uncertain, as is its generality. However, in 1974, Risser and Pollack observed similar patterns of expression in unselected populations of 3T3 cells infected with SV40 (*Virology 59:* 477 [1974]). Moreover, we have observed certain "ambivalent" phenotypes in the expression of the endogenous *tk* gene in avian and rodent cells, which are consistent with rapid switching (see below). Evidence from blot hybridization suggests that switching is not due to large-scale sequence rearrangements, and so other mechanisms are now beginning to be studied. The following major questions can be asked: Is switching peculiar to SV40? Does it occur coordinately to several independently integrated transcription units? Is the constitutively switched-on state (which occurs in a minor proportion of independent transformants) due to a host mutation or to an SV40 mutation, or is it due to a mutation at all? Is switching correlated with changes in DNA methylation patterns?

In a manner similar to that employed in our SV40 studies, both the cloned chicken and herpes *tk* genes were introduced into doubly mutant mouse LTk^-Aprt^- cells using *aprt* as the selection system. As judged by the ability of cotransformants to grow in *tk*-selection (HAT) medium, all cell populations that contained either the herpes *tk* 3.4-kb *Bam*HI fragment or the 2.25-kb *Eco*RI/*Hin*dIII fragment of chicken *tk* were capable of growing in HAT. Thus, these genes are completely encoded on their respective fragments. Work in progress indicates that the pattern of expression of the chicken *tk* gene in mouse cells resembles the

expression of mouse *tk* in so far as levels of the gene product are greatly reduced in stationary cell cultures. If this regulation is at the level of transcription, it may be possible to identify the control region for modulation of expression. Neither in the case of the herpes *tk* gene nor that of the chicken *tk* gene have we studied expression at the single cell level as we did for SV40, and so our results are not comparable. Preparations for such studies, based on the construction of chimeric genes, are in progress. Future studies include the construction of chimera between selectable genes and genes under differential control in order to apply the techniques of mutation and selection to obtain information about the mechanisms of cellular differentiation.

Characterization of the Chicken *tk* Gene

K. Kwoh, M. Goldfarb, M. Perucho, M. Wigler

As described in last year's report, we have isolated the chicken *tk* gene by the technique of plasmid rescue. The gene has been cloned both as a 2.25-kb *Eco*RI/*Hin*dIII fragment in pBR322 and as a 15-kb clone in the λ vector Charon 4A. As described in the previous section, we believe that the 2.25-kb fragment is complete—encoding information for its own transcription and regulation. Our aim is to provide a complete anatomical description of this gene, and to this end we are sequencing it, have made an extensive collection in vitro of pseudo-random linker (*Xho*I) insertion mutants, and have a preliminary characterization of the transcription unit—its size, splicing pattern, and orientation.

Characterization of Tk Mutants in Cultured Chicken Cells

M. Wigler

We have obtained a permanent chicken cell line courtesy of G. Shutz and T. Graf (University of Heidelberg, Germany). Called 249, it is derived from an MC29-induced hepatoma in chickens. The availability of this line and the cloned chicken *tk* gene has made possible a molecular investigation of mutations that arise at the *tk* locus after selection of cells resistant to killing by BrdU. 249 cells plate in medium containing BrdU at about 5 colonies per 10^8 cells plated. It is possible to select mutants in that way or by gradually selecting cells resistant to ever-increasing BrdU concentrations. We have used both approaches to obtain 10 independent BrdU-resistant mutants. All the mutants that have been analyzed to date appear to have a normal blot hybridization pattern for *tk* but one of reduced intensity relative to the 249 parent, suggesting that one step to BrdU resistance has been deletion of all *tk* sequences from one locus. A similar deletion of the remaining *tk* locus has not occurred in any line and is presumably lethal. The second step appears to differ in various mutants, since there are at least two distinct phenotypes: mutants that cannot grow in HAT medium (plating efficiencies less than 10^{-6}) and mutants that grow in HAT medium with high efficiency. The latter class appears analogous to the cells that demonstrated heterogeneous expression of the SV40 early region as discussed earlier, in that efforts to clone a pure population have so far failed. Work is currently directed at determining whether the ambivalent phenotype does represent rapid switching and, if so, whether this phenotype can be regarded as due to a mutation at the DNA sequence level.

Isolation of Human Tumor Genes

M. Perucho, M. Goldfarb, K. Shimizu, M. Wigler

Recent work of R. Weinberg's laboratory (MIT) and G. Cooper (Sidney Farber Cancer Institute) has demonstrated the feasibility of transforming normal-growth-controlled rodent cells to the tumor phenotype using DNA from chemically transformed or virally transformed cells as donor. These experiments, together with our ability to clone the active gene by plasmid rescue, raise the possibility of a genetic analysis of tumorigenesis. We have begun screening DNAs from a variety of cells derived from various human tumors for their ability to serve as donors of the tumor phenotype using NIH-3T3 cells as the normal recipient. Several human cell lines have been identified that are excellent donors of the tumor phenotype, and we are now attempting to isolate these genes for further study. The techniques we will employ involve plasmid rescue, which we used to isolate the chicken *tk* gene, and a new approach utilizing a bacterial tRNA suppressor gene as the prokaryotic factor. The latter will complement a λ packaging phage containing an amber mutation in the lysis function. Only recombinant phage will be able to grow on wild-type hosts.

Inheritance and Function of DNA Methylation

D. Levy, M. Perucho, M. Wigler

The DNAs of higher eukaryotes contain a low proportion of modified cytosine residues (5-methylcytosine [5-MeC]), whose function has been the subject of much speculation and interest. Most of the 5-MeC residues occur in the simple palindromic sequence

5′ CpG
3′ GpC

and this sequence is also found as part of the recognition site of several restriction enzymes that will not cleave methylated recognition sequences. By using such "CpG" enzymes, many workers

have investigated the distribution of methylation within specific gene sequences in specific tissues. To a first approximation, methylation patterns are not random: There appears to be a master pattern, present in germ cells, of which the somatic cells partake, the exact variation in methylation pattern differing between tissues but being characteristic for particular tissues. This distribution is consistent with the idea that methylation patterns are inherited by somatic cells. Moreover, there is a rough inverse correlation between degree of methylation and expression, consistent with the idea that methylation patterns might function in some way to control specific expression patterns in differentiated tissues. We have decided to explore these questions using the methods of gene transfer. We purified M-*Hpa*II, the methylase from *Haemophilus parainfluenzae* that methylates the internal cytosines of the sequence

5′ CpCpGpG
3′ GpGpCpC

a subset of potentially methylatable sites in higher vertebrates. Using M-*Hpa*II, we demonstrated that methylation patterns established on cloned DNA in vitro are replicated in vivo when that DNA is introduced into living cells. In other words, methylation is its own genetic determinant. We estimate that the fidelity of inheritance is at least 95% per site per cell generation. Moreover, we observed that the methylated herpes or chicken *tk* genes transform cells less efficiently than unmethylated genes, indicating that there is a causal relationship between expression and methylation.

Publications

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