Special Achievement in Medical Science Award

The surprises of mammalian molecular cell biology

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The opportunity provided by the Albert Lasker Award for Special Achievement in Medical Science to reflect on my 'career' does not, I am sure, give me license to dwell on the humid, sweltering summers of a childhood in Mississippi. But perhaps I may be permitted to say a brief word of thanks to my teachers at the University of Mississippi, especially Dean Parker, a *Drosophila* geneticist of the T.S. Painter School, and Robert Glaser, at the time an assistant professor of medicine at Washington University School of Medicine. Parker taught us what a gene was and how to observe its effects, but it was in Robert Glaser's rheumatic fever laboratory that I tried to do my first experiments. A colleague, Stephen Morse, and I infected rabbits in their tonsils with Group A streptococci in an effort, not completely unsuccessful, to mimic the secondary heart muscle damage that occurs in rheumatic fever as a sequel to infection by Group A streptococci[1].

After a year of internship, I had my introduction in 1956 to basic biomedical research in Harry Eagle's laboratory at the National Institutes of Health in Bethesda, Maryland. Eagle had worked on penicillin previously, but by the time I made it to Bethesda, he had developed a defined medium for reproducible propagation of mouse and human cells in culture, and had left behind his studies on penicillin. Eagle's work made homogeneous populations of human cells available for biochemical and virological studies. Perhaps because Eagle thought I had an abiding interest in infectious disease, it was strongly hinted that I should work on viruses. Good fortune can also favor the totally unprepared: I knew alarmingly little of viruses, but shared a room in the Eagle laboratory with Robert I. DeMars (Fig. 1), who had been the third graduate student of Salvador Luria, the great bacteriophage geneticist and, of course, a key member of that small band of founders of molecular biology. DeMars handed me his copy of the first edition of Luria's *General Virology* (1953) and said that if I read it, he would teach me virology. I did, and he did, or at least he tried to. My first charge was to develop a plaque assay for poliovirus using HeLa cells, one of the continuous cultures in common use in the Eagle laboratory. I then demonstrated that each cell could be infected by showing that each could plate as an infectious center on a lawn of uninfected cells, and I finally described a one-step growth curve for poliovirus[2]. Thus I was following my 'phage mentor' precisely.

With Leon Levintow (Fig. 1), a biochemist in the Eagle laboratory, my aims turned to charting the course of virus RNA and virus protein formation to compare this with infectious virus formation. Jerome Vinograd, the father of equilibrium density gradient centrifugation using cesium salts, visited Eagle's laboratory and told us about his work. We were soon able to purify poliovirus conveniently using the equilibrium density gradient technique. By labeling infected cultures with either amino acids or adenosine at various intervals after infection and then purifying the cultures and measuring the radioactivity in the virus at the conclusion of one virus cycle, we could chart how much virus protein or RNA had been made at the time the label was added. We could then deduce the 'growth curve' of the virus constituents to compare this with the growth curve of the infectious virus particles obtained from disrupted cell samples[3,4]. This was in 1959, before the idea of messenger RNA (mRNA) was born. I was intrigued with the finding that poliovirus RNA was made before poliovirus protein and wondered what it meant. I knew during this time I should eventually leave 'the Eagle's nest', and Bob DeMars suggested that my next step in scientific education plus cultural enlightenment (equally necessary) was to go to Paris and work with Francois Jacob and learn genetics. Eagle agreed that this was a splendid idea and offered to send me and provide me at least a semi-permanent position upon my return to Bethesda. Thus I applied to Andre Lwoff, Chef de Service de Physiologie Microbienne at the Pasteur Institute, in whose service Jacob worked. On one gloriously happy June day in 1959, I received a letter of acceptance from Lwoff.

However, before I left, Salva Luria, who was at that time (1958–1959) moving from Illinois to the Massachusetts Institute of Technology (MIT), offered me an opportunity to join the MIT faculty. When I told him I desperately wanted to join Jacob at the Pasteur for my year there, Luria somehow wangled a postdoctoral salary from an MIT training grant to pay me to go to Paris so that I could return and join the MIT faculty as an assistant professor. The year in Paris was a revelation in many ways to this 29-year-old Mississipian. When I arrived, Francois was working with Jacques Monod on the famous *Journal of Molecular Biology* paper[5] explaining to the world how gene regulation occurred through mRNA synthesis in *Escherichia coli* and its phages. Although I went to Paris to learn genetics, much of my time was spent teaching people how to pour sucrose gradients and separate RNA molecules, which were naturally 'all the rage' in the Paris laboratory that year. During that year I wrote my first National Institutes of Health grant to establish the first cell culture/animal virology

Fig. 1 Robert I. DeMars (left), Leon Levintow (right) and J.E. Darnell (center) in 1956 in Harry Eagle's laboratory at the National Institutes of Health. Courtesy of Leon Levintow.

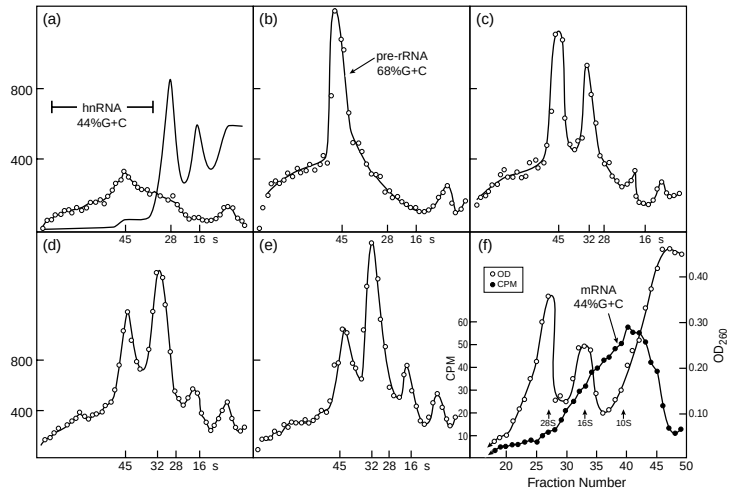

Fig. 2 Sedimentation and base composition analysis of HeLa cell nuclear RNA and polysomal mRNA. ***a–e***, RNA was extracted from HeLa-cell nuclei after 5 (*a*), 15 (*b*), 30 (*c*), 45 (*d*) or 60 (*e*) min of labeling. Sedimentation values were estimated from two known ribosomal RNA values (28S and 18S). The hnRNA profile (about 16–60S) is dominant at 5′ and is low in G+C (44%), like cell DNA. The dominant peak at 45S is high in G+C (65–68%) and represents pre-rRNA. ***f***, Sedimentation analysis of RNA obtained from polyribosomes after brief labeling with ^{32}P. Base composition of labeled mRNA (—|—), 44% G+C. *a–e*, Adapted from refs. 41, 7, 42. *f*, Adapted from ref. 9.

laboratory at MIT, and was told flatly by Luria I was expecting much too much monetary support. But I argued with him that I had carefully noted what Eagle's laboratory used and how much I expected to spend to run an animal cell laboratory, and sent in the grant unchanged. The National Institutes of Health came through with every penny, starting what has been generous support for over 40 years.

As I had by this time learned to extract poliovirus RNA in a single infectious piece and had 'pulled' the RNA out of bacteria intact, I was ready to start examining the RNA of the human HeLa cell. My first postdoctoral fellow, Klaus Scherrer, and I succeeded in developing a strategy (hot phenol plus sodium dodecyl sulfate) that extracted nearly 100% of the RNA. We subjected the RNA to size separation on sucrose gradients and found immediately that the RNA most rapidly labeled (within 5 minutes) was entirely in the nucleus and was heterogenous in sedimentation profile, in contrast to the ribosomal RNA and transfer RNA (tRNA) in the cytoplasm[6] (Fig. 2). Only after 15–20 minutes of labeling did the newly labeled nuclear RNA show a sharp peak. However, this peak was about 12,000–14,000 nucleotides in length, much larger than the 2,000 and 5,000 nucleotides in the two ribosomal RNA molecules. We then labeled for a short period of time, stopped all further RNA synthesis by adding actinomycin, a DNA-binding agent that stops RNA synthesis, and found that the large nuclear peaks shifted to the size of ribosomal RNA. Our only means of characterizing the nature of these RNAs at this time was to examine their base composition using ^{32}P-labeled RNA. The specifically sized (14,000-nucleotide peak) large nuclear RNA was high in guanine and cytosine residues (G+C), as was ribosomal RNA (ref. 7). We had discovered pre-ribosomal RNA, the first RNA processing to be discovered. Later pre-tRNA was also identified[8].

The big remaining mystery was the nature of the very large, rapidly labeled, heterogeneous nuclear RNA that had an average base composition of 42–44% G+C, like that of all vertebrate DNA. This DNA-like RNA was called heterogeneous nuclear RNA (hnRNA). While these experiments were continuing on the eighth floor in our laboratory at MIT, Jon Warner and Paul Knopf in Alex Rich's laboratory one floor below had discovered polyribosomes, a cluster of ribosomes thought to translate a single mRNA. When we isolated briefly labeled RNA from HeLa cell polyribosomes, it was DNA-like and heterogenous (about 500 to 2,000–3,000 bases in length), not at all like ribosomal RNA (ref. 9). We assumed this to be mRNA for, among other reasons, when poliovirus infected cells, host protein synthesis was halted and host polysomes disappeared, to be replaced by polysomes that contained virus RNA acting as mRNA. Was the DNA-like hnRNA a precursor to the polysomal mRNA, as apparently were the large pieces of RNA in the nucleus precursors to ribosomal RNA? From this time in 1963 through the succeeding 14 years, we tried to link hnRNA to mRNA or to refute the possibility that they were linked.

In these early times we had no way to identify specific cell mRNAs, so we turned to DNA viruses, which afforded us the opportunity to study individual mRNAs by hybridizing mRNA to viral DNA. The DNA of simian virus 40 (SV40) enters the cell nucleus and becomes integrated into cell chromosomes, but SV40 mRNA is still produced from the integrated chromosomal locus. We found the SV40-specific nuclear RNA ranged in size from 8,000 to 15,000 bases, whereas the SV40-specific mRNA from the polysomes ranged from 1,000 to 5,000 bases, clearly in line with the possibility that RNA was being processed to make mRNA (ref. 10). But the result was only indicative; we still needed some 'tag' on the cellular hnRNA that might appear in mRNA. Such a 'tag' appeared in 1970–1971. Several laboratories (Edmonds, Brawerman, Kates) in addition to our own found that a segment of adenylic acid residues, or 'poly(A)', was present on most if not all mRNA isolated from polyribosomes[11–14]. My experiments originated from the isolation of polysomal RNA from cells labeled with either adenosine or uridine and the finding that ribonuclease A completely digested the uridine-labeled RNA but left a core of undigested adenosine-labeled RNA. We then showed that the same 'chunk' of adenosine-labeled RNA existed in hnRNA and, most importantly, when the label time was restricted to a few minutes, almost all of the poly(A) was in hnRNA, appearing only later in polysomes. Moreover, the poly(A) was initially exactly the same size in the hnRNA and in the polysomal mRNA (ref. 15) and both existed at the 3′ end of the RNA molecules. This convinced us, if not the rest of the world, that at least a part of the hnRNA (the 3′ end) was processed into mRNA. We turned back to viruses to pursue this question farther. Adenovirus, like SV40, is a DNA virus whose DNA enters the cell nucleus to be copied into virus mRNA. Moreover, it is easier to grow adenovirus than SV40 in cultured cells. So we had begun to study adenovirus when Lennart Philipson, an old friend and virologist from Sweden, came to our laboratory at Columbia University for a sabbatical. We soon showed that adenovirus mRNA also had poly(A) the same size as that of the cellular mRNA added in the nucleus[16]. Furthermore, the adenovirus-specific nuclear RNA was much larger than the adenovirus-specific polysomal RNA. After we moved the laboratory to Rockefeller University, we mapped (by pulse labeling techniques and ultraviolet transcriptional mapping) the late adenovirus RNA start site and demonstrated that primary transcripts of adenovirus RNA were 30 kb in length[17,18].

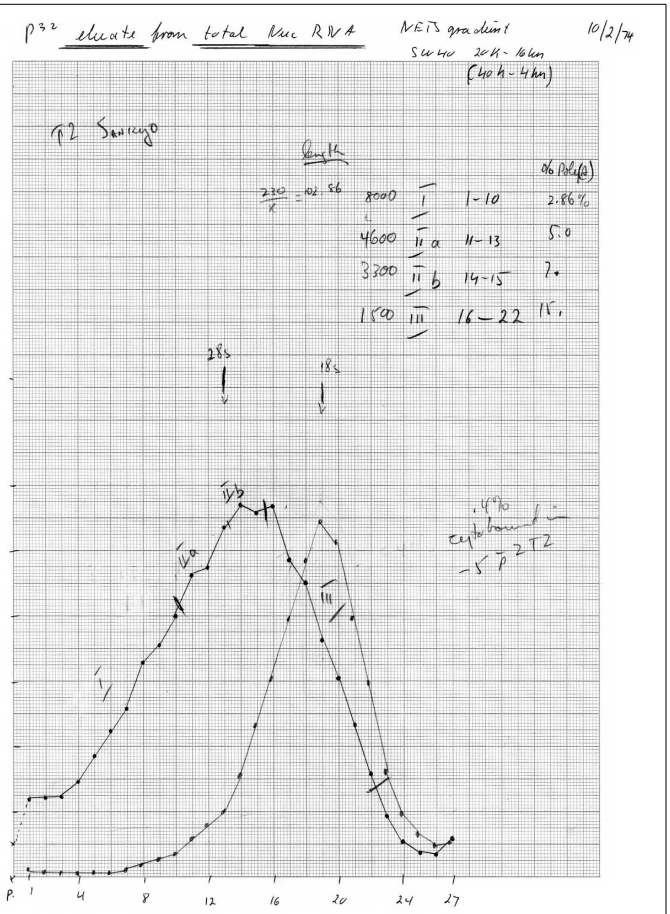

Fig. 3 Analysis of ^{32}P-labeled poly(A)-selected hnRNA and polysomal mRNA. Graph, from experiment of 2 October 1974, Marianne Salditt-Georgieff's notebook shows larger hnRNA and smaller polysomal mRNA. Size is indicated by 28S and 18S rRNA labeled with ^{3}H and sedimentation is right to left. Each sedimentation fraction (I–III) for hnRNA and all the mRNA was analyzed for poly(A) content and cap content. The percent of each sample that was poly(A) (about 230 nucleotides in length) was used to calculate chain length as entered in the table. The caps (^{7}mG*P*XmpXp) were collected and redigested, leaving m^{7}GpppX, and the data were used to calculate chain length. Cap content calculation showed the largest fraction was 10,700 nucleotides (compared with 8,600, based on poly(A)), whereas the mRNA was 1,370 nucleotides. Thus, each molecule in each size class contained a cap and poly(A). Why did we not see that to derive mRNA splicing was required?

The final step in discovering biochemical markers for mRNA in mammalian cells was addressed to the 5′ end of the molecule. Perry had shown methyl label transfer into mouse cell polysomal mRNA (ref. 19), and several laboratories had found that the 5′ end of both animal viruses had a 5′ methylated 'cap' structure—m^{7}G*P*XmpXp (ref. 20). One of these laboratories was headed by Aaron Shatkin, a long-time friend who had worked in the Eagle laboratory just after I left. Together with Aaron's laboratory, my technician Marianne Salditt-Georgieff and I showed that caps existed in HeLa cell polysomal mRNA as well as in HeLa cell hnRNA. Once again the mRNA 'decoration' appeared first in hnRNA. Finally, we showed with Aaron's group[21] that hnRNA selected to contain poly(A) also contained a cap. The cap had 5 phosphates and the poly(A) had about 200 phosphates, whereas the hnRNA had up to at least 10,000 phosphates in the hnRNA molecules of various sizes. On the other hand, the molecules of capped, polyadenylated polysomal mRNA averaged 1,500 nucleotides in length (Fig. 3). Our conclusion, logical but wrong, was that mRNA must be fashioned from each end of the long hnRNA molecules. We lacked the vision or imagination to propose that the cell discarded the middle of the molecule and rejoined the ends, a bit of molecular 'carpentry' now known as splicing. Thus, our work had set the stage for the discovery of splicing, but it was the beautiful electron microscope pictures from two groups, Phil Sharp and his colleagues at MIT (ref. 22) and a large group of young scientists at Cold Spring Harbor[23], that demonstrated this radical fact of nature. As some students of molecular biology do know, and all should know, both groups showed that adenovirus mRNA contained 5′ segments arising from the transcription start site we had mapped plus parts of the long adenovirus specific RNA transcript, with the loss of the intervening sequences. The primary transcript had been cut and pieces rejoined to make adenovirus mRNA. A flood of additional experiments followed showing that splicing also occurred in many different cellular mRNAs, and of course it is now known to occur in the production of most mRNA from animal cells.

Although it was enormously satisfying that the problem of mammalian mRNA biogenesis finally had been solved, the question of mRNA regulation was only beginning. By the late 1970s, the techniques of copying mRNA into DNA and amplifying it in bacteria had become routine. With ample DNA complementary to individual mRNA sequences (cDNA), it finally became possible to measure directly the relative rate of transcription of individual genes by labeling nuclear RNA and hybridizing it to the individual amplified cDNAs. Thus we could now turn to the question that motivated our study of mammalian RNA in the first place: the regulation of individual genes. We made copies of rat liver cDNAs and selected those that proved to be either absent or present at very low levels in spleen or kidney[24,25]. The labeled nuclear RNA from liver cells hybridized to 12 different liver-specific cDNAs, but the labeled nuclear RNA from kidney or spleen nuclei did not. The mRNAs common to all cells (such as actin and tubulin) were made by all nuclei. Thus, differential transcriptional control was responsible for the production of liver-specific mRNAs.

What signaled a cell to express particular genes? When we disaggregated liver cells (hepatocytes) and put them in culture, they very quickly lost the liver-specific transcription profile but were still alive, making proteins that were specific for liver on the preexisting mRNAs (ref. 26). We concluded that signals from the outside were disrupted in taking the liver cells apart, indicating that signals from the outside maintain transcriptional control in the nucleus. Nothing was known at that time about how signals from the cell surface could be passed to the nucleus and affect transcription.

We sought to study a situation in which an extracellular protein might change the transcription rate of genes, and chose to study interferon (IFN). IFN caused cells to enter an antiviral state within an hour or two, and new RNA and protein synthesis were required for this conversion[27]. Moreover, my friend and former colleague, Ernest (Pete) Knight had purified IFN and offered a sample for study. cDNA copies from IFN-treated and untreated cellular mRNA were prepared by Andy Larner, a postdoctoral fellow; he found two cDNAs that hybridized only to the mRNA from treated cells[28]. Moreover, only isolated nuclei from treated cells and not untreated cells produced labeled nuclear RNA complementary to these cDNAs. Thus, we had identified genes that were transcriptionally induced by IFN.

From 1984 until 1992, a steady progression of experiments followed to explain the basis for this induction[29,30]. DNA segments upstream of the IFN-stimulated cDNA sequences were capable of directing IFN-dependent transcriptional control in transfection assays. Specific sites within these putative regulatory regions bound proteins that preexisted in the cell but were converted by IFN treatment into active DNA-binding proteins. The binding sites (about 15 base pairs in length) were similar in two different genes[29]. Purification of the binding proteins yielded material that, with the use of modern techniques, allowed peptide sequence analysis, leading to the cloning of the induced DNA-binding factors[31]. Two proteins were identified that were both phosphorylated on a single tyrosine in response to IFN-α but only one of which was tyrosine-phosphorylated in response to IFN-γ. After activation, the proteins enter the nucleus, where they bind DNA and activate transcription. Because the proteins were both signal transducers and activators of transcription, we called them STAT1 and STAT2 (ref. 32).

A year later, STAT3 and STAT4 were discovered by two graduate students, Zhong Zhong and Zilong Wen[33,34]. It is now known that there are seven STATs in human cells and that these proteins are involved in many crucial developmental pathways and in maintaining homeostasis in adults[35].

Of central present-day interest is STAT3. Beginning in the mid-1990s, clinicians reported the presence of activated DNA-binding complexes without an obvious stimulus from outside the cell to cause STAT activation[36]. Moreover, STAT3 was persistently activated in all head and neck cancers and all multiple myelomas and in many other tumors as well[37]. We showed that it was possible to make a persistently active STAT3 molecule and that this molecule was oncogenic[38]. In tumors, this constitutive overactivity of STAT3 protects the tumor cell from apoptosis[39]. Cancer patients should benefit when the pharmaceutical industry successfully takes on the difficult but possible task of finding small molecules that block protein–protein associations so that constitutive activity of transcription factors such as STAT3 can be controlled[40].

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