My Life with Adeno-Associated Virus: A Long Time Spent Studying a Short Genome

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My 45 years of studying the molecular biology of adeno-associated virus are recounted. Additional activities as a mentor, department chair, and medical school administrator are described, as are my activities in the public sphere, which involved national issues related to science policy and medical education.

AS I ALWAYS told my students, all other things being equal, it is better to be lucky. I consider myself to have been extraordinarily lucky with respect to my family (parents, spouse, sister, and children), my education, and the professional opportunities I have had. My good luck has held true for my research career as well. Forty-five years ago, I began to study adeno-associated virus (AAV). It was interesting to me because of its unusual life style and its small size. However, because it caused no known disease and required coinfection with an adeno- or herpesvirus for a productive infection in cell culture, skeptics wondered why I was wasting my time working with it and suggested that AAV really stood for “almost a virus.” Of course, time has shown that the joke was on the critics; today, AAV is one of, if not the most widely used vector for gene therapy. Success in using the virus in this way has required a detailed understanding of the molecular mechanisms underlying the biology of the virus. This is a classic example of the value of basic research leading to applicability in the real world.

My first research experience was after my junior year at Harvard in Paul Doty’s laboratory working with Julius Marmur to study UV irradiation of DNA. I did two projects; the first was to see whether sensitivity to UV damage was related to molecular weight (MW) of the DNA and indeed it was. The second project was to see whether DNAs with differing GC contents were differentially affected by UV as reflected in a decrease of the melting temperature (T_m). The results clearly indicated that the decrease in T_m was directly proportional to the AT content of the DNA. Within the year, it was appreciated that this result supported the finding by Dutch investigators that the major lesion caused by UV irradiation was the thymine dimer (Marmur *et al.*, 1961).

In the fall, I entered the new Year I program at Johns Hopkins; it was designed as a 2/5 program, but since I had already had 3 years at Harvard, I did not have to take many

formal courses and instead spent almost all the time working in the laboratory of Charles A. Thomas. My project was to see if I could extract the DNA of T4 bacteriophage as a single molecule. Using biologically active cell walls, we were able to do so. The phage attached to the cell walls and injected their DNA through the wall into the solution. Unfortunately, the methodology for measuring MW of such large molecules was just being developed; we measured the variance of the width of the bands the DNA formed in CsCl gradients in the analytical ultracentrifuge. The analysis indicated the MW of the DNA to be at least equivalent to half the genome (Thomas and Berns, 1961). Later, Irwin Rubinstein, a postdoc in the Thomas laboratory, used autoradiography to show that the DNA we had extracted indeed did represent the intact genome. A related finding was that the T4 genome double helix was composed of two continuous single polynucleotide chains (Berns and Thomas, 1961). This finding seems intuitively likely now, but in those days, the notion that DNA with a MW of 120 megadaltons consisted of two DNA single strands with no linkers of protein or other chemicals was difficult to comprehend.

My experience in the Thomas laboratory was so stimulating that, after the first regular year in medical school, I took leave of absence to attend graduate school. I was a student in the Biology department, but I did my thesis research in the Thomas laboratory in Biophysics. The course work gave the theoretical basis underlying many of the techniques I used, so it was quite satisfying. My research was to see if I could extract bacterial DNA that was much larger than had been achieved before. I worked with *Haemophilus influenzae*, which has a relatively small genome and was successful in extracting DNA that was much larger, greater than 300 megadaltons (Berns and Thomas, 1965). This size represented about half the genome. The large size was supported by the observation of genetic linkage between markers that had appeared unlinked earlier in assays of

bacterial transformation (work done in collaboration with the laboratory of Roger Herriott in the School of Public Health) and by measurement of the DNA in the electron microscope by Lorne MacHattie in the Thomas laboratory.

After finishing my thesis, I married Laura and returned to finish medical school, which I was able to do over the next 22 months, thanks to skipping normal elective quarters and summers off. I did a pediatrics internship at Hopkins for three reasons: I liked the department, I knew that genetics is significant in pediatrics, and I thought it would give me useful information when we had kids. With regard to the last reason, mostly it scared the dickens out of me. It was a great experience, second only to my first year in the Thomas laboratory.

All of my education at this time was done during the Vietnam war; almost without exception all physicians had to serve. Fortunately, one alternative to going into the military was to gain a slot at the National Institutes of Health (NIH) in the U.S. Public Health Service. I was fortunate enough to get such a slot in the laboratory of Arthur Weissbach. Art was a DNA enzymologist and we decided that I should work with Vaccinia virus. That allowed Art to transition from lambda phage and me to get experience in enzymology and with an animal virus. Norman Salzman's laboratory was just down the hall and he supplied us with enough of the virus and the materials necessary to grow it to get us going. During that year, I was able to separate the Vaccinia DNA-dependent DNA polymerase from the cellular DNA polymerase and to partially purify and characterize the Vaccinia enzyme (Berns *et al.*, 1969). At the end of the year, Art left the NIH for the new Roche Institute of Molecular Biology and I transferred into the Salzman laboratory. There I continued to work with Vaccinia, and Carol Silverman and I discovered that the complementary strands of the DNA were cross-linked (Berns and Silverman, 1970). A couple of years later, after I had returned to Hopkins as a faculty, my student Paul Geshelin did some very elegant electron microscopy, which showed that the crosslinks were at the ends of the DNA and were most likely simple DNA hairpins (Geshelin and Berns, 1974). Eventually, Bernie Moss demonstrated that this was indeed the case.

A critical event in my career was meeting Jim Rose, a senior investigator in our overall group, who introduced me to AAV. After we had a good interaction at a laboratory party, Jim came to me with a problem; he and Aaron Shatkin had just published an article showing that purified AAV DNA was a linear double helix with a MW of 3.6 megadaltons. Suddenly an article from Lionel Crawford's group in London appeared that suggested that the AAV genome was actually a linear single-stranded DNA with half the MW of the purified DNA. Lionel suggested that an unlikely possibility was that half of AAV virions contained a plus strand and the other half a minus strand, so that when the DNA was extracted, the complementary strands would anneal to form the double-stranded DNA Jim and Aaron had observed. Jim wanted to know how to test the hypothesis. The next day I suggested the following experiment: if an AAV virus preparation containing heavy-density DNA labeled with BUdR was mixed with an AAV virus preparation containing unlabeled DNA of normal density and the DNA extracted from the mixture, the purified DNA should have an intermediate density in a CsCl gradient. This proved to be

the case (Rose *et al.*, 1969); thus, Lionel's unlikely suggestion turned out to be true.

Although I had the chance to stay at the NIH on a permanent basis, I decided to return to academia. Fortunately, Barry Wood, who was responsible for my originally coming to Hopkins in the Year I Program, which he had started, offered me a position in the Microbiology department. Dr. Wood also arranged for my appointment as a Hughes investigator. My laboratory had Dan Nathans on one side and Ham Smith on the other. Smith, Kelly, and Wilcox had just published the characterization of the target sequence for the HindIII restriction enzyme. Nathans was on sabbatical in Israel with Ernest Winocour to learn to work with SV40; he came back to his classic studies on the SV40 genome using Ham's restriction enzyme. Consequently, my laboratory had a lot of strong stimuli. As described earlier, Geshelin worked with Vaccinia and the rest of us worked with AAV to characterize the DNA, including making physical maps of the restriction fragments and characterizing the ends. Ken Fife, a student studied the very ends of the genome, along with Hugh Gerry. Every time Hugh tried to label the 5' nucleotide, he separated a mixture of nucleotides. Some were G, others more like T. Finally, Fife and I set up a collaboration with Ken Murray in Edinburgh to use the wandering spot technique to sequence short stretches of the 5' termini. The data obtained in Murray's laboratory were at first confusing, but finally, it was appreciated that there were 3 terminal sequences, one terminated with G, one had a single T to the 5' side of the G, and the third had two T's 5' of the G. So, all of the heterogeneity at the 5' termini were at the level of three nucleotides (Fife *et al.*, 1977). This was typical of the challenges involved in studying the ends of AAV; the issue was complicated, because as we subsequently showed, the ends of the DNA were palindromic inverted terminal repeats with an additional 20 nucleotides in the inverted repeat beyond the palindromic region (Lusby *et al.*, 1980). The overall palindrome contained two additional palindromes, one on either side of the middle nucleotide in the overall palindrome. Thus, when the overall palindrome was folded on itself, a T-shaped structure was formed containing 125 nucleotides. There were only seven unpaired bases, three at the tip of each cross arm of the T and one separating the two cross arms. Toward the end of my 6 years in the Microbiology department, Ham Smith returned from a Gordon conference, where the Maxam-Gilbert method of DNA sequencing had first been presented; immediately, Ken Fife began to use it to sequence AAV, beginning at the ends. By this time, the effort of graduate students Hugh Gerry and Ilene Spear had led to a good physical map of the genome (Spear *et al.*, 1977). Eventually, 6 years later, we published the entire AAV2 DNA sequence (Srivastava *et al.*, 1983).

In 1973, I was offered the chance to direct the Year I program, the program that had brought me to Hopkins. Characteristically, I could not say no. So, I wound up with 30 undergraduate advisees and became the only nonchairman in the School of Medicine to be responsible for an academic program. I was also considered a member of the dean's staff. Since the Year I students spent considerable time on the Homewood campus, I became the medical school liaison to that campus, including on the graduate council. Fortunately, despite my junior status, my life was made easier by the fact that the Homewood dean was a good friend, Sig Susskind,

from Biology, who had served as my cothesis advisor. Dealing with friends makes academic administration much easier.

In 1976, unexpectedly, I was offered the chair in Immunology and Medical Microbiology at the University of Florida College of Medicine in Gainesville. By that time, Dan Nathans had become the chair in Baltimore and I kept telling him what to do; often he would actually do it. Therefore, I thought that either I should accept the Florida job with its responsibilities or keep quiet. Since the latter seemed unlikely, I decided to go to Gainesville. The decision was made somewhat easier because Laura's sister was living there as her husband Charles Mahan was on the OB/Gyn faculty. Dick Ross, then Hopkins dean, made a nice counter offer to be his deputy, but it would have meant giving up much of my research time and I was not ready to do that. Over the years, Dick and I became good friends.

I arrived in Gainesville to find that I was the youngest faculty member in the department (I was 38). We had an initial faculty meeting where I set out my goals for the department. All went well until I said that one of my goals was to double all of their salaries over the next 2 years; that was greeted with an uproar of laughter and comments that I would learn that much of the pay in Florida was in sunshine. Anyway, the thought was appreciated and, when it actually happened, my stock was certainly enhanced. In addition to trying to set up a laboratory, much of my time was spent in recruiting new faculty. Everyone pitched in to help and we recruited several outstanding young people, most of whom are still in Gainesville in senior positions. Of the first five recruited, three are now in endowed chairs and a fourth is the chair of Biochemistry. The fifth followed me to New York eventually and has been a full professor at Cornell for the past 25 years. My interest in becoming a chair was to see if I could create an academically positive environment in which students and young faculty could flourish. In this effort, I was successful. We continued the excellent, existing departmental graduate program. The students we admitted had good, but not great academic records. Our success stemmed from the fact that we made the students give presentations from the very beginning. First year students gave six talks to the faculty, three describing projects they were going to do and another three describing the results. This pattern continued throughout their training. They were required to have an external examiner when about one third of the way through their research and often received offers to do a postdoc with the examiner. The major differences for me as a chair were the close involvement in the direction of the College of Medicine and the responsibility for the well-being of all the faculty and students. Support internally from the faculty was excellent, the other chairs were cooperative and the dean supportive. It was a very positive experience.

During this period, we continued to work with AAV. We identified the initiation site for DNA replication, defined the viral genes, mapped the start points of transcription of the early genes, and worked on integration of AAV (Hauswirth and Berns, 1977; Cheung *et al.*, 1980; Lusby and Berns, 1982). Bill Hauswirth, who went from being a postdoc in the laboratory to a faculty member, was a key player. One of our first faculty recruits was Nick Muzyczka, who had come from Dan Nathans' laboratory committed to SV40. However, a mutual grad student Jude Samulski served as a link to

draw Nick into the AAV orbit. Mark Labow, Roy Bohenzky, Jeff Ostrove, Andy Cheung, Ed Lusby, and Mark Rayfield were all successful contributors (Ostrove *et al.*, 1981; Bohenzky *et al.*, 1988). Silvano Riva, a visitor from Italy, and postdoc Rance Lefebvre showed that the secondary structure of the AAV terminal repeat was critical to the ability to be rescued from the plasmid construct and replicated (Lefebvre *et al.*, 1984). A key contribution was Samulski's cloning of the intact AAV genome into a biologically active plasmid from which, wild-type virus could be produced after transfection into human cells (Samulski *et al.*, 1982). This opened the way for detailed genetic analysis.

During my time as chair, Robert Marston was the university President. Bob had come to Gainesville after being fired as Director of the NIH by Richard Nixon for too publicly supporting the NIH budget (Bob told me that was one of his proudest achievements). Soon after my arrival, the then dean Al Stetson took me to meet with Bob. It was a cordial meeting at the end of which, Bob told me that my mandate was to bring modern biology to the University of Florida. I did try to do some of that in our own department and in collaborative efforts with agriculture. The university was certainly successful in the effort over the years; I like to think that I contributed.

About 1980, recombinant DNA became a hot topic, and fears about potential negative environmental consequences were increasingly expressed. As a result, there was the threat of federal legislation to restrict such research. Major research universities mounted a public relations effort to keep any such legislation from having a chilling effect. Bob Marston asked me to participate as Florida's representative. It was an unusual personal situation, because almost all others involved were public affairs people from places such as Harvard and Stanford. As the only working scientist in the group, I became the "expert" on technical issues. I was also selected to be the representative of the Association of Medical School Microbiology and Immunology Chairs to the Council of Academic Societies (CAS) of the Association of American Medical Colleges (AAMC). At the same time, I joined the Public and Scientific Affairs Board of the American Society for Microbiology (ASM). A final consequence was that, I became an early member of the NIH Recombinant DNA Advisory Committee and eventually the chair.

In 1982–1983, I did a sabbatical in Ernest Winocour's laboratory at the Weizmann Institute in Israel. Ernest was a noted SV40 geneticist who had introduced Dan Nathans to SV40. Ernest was interested in the ability of SV40 to recombine with heterologous sequences. My project was to study recombination between SV40 and AAV. After a week or so in the laboratory, one of the grad students Zehava Grossman told me that she wanted to work on the project for her thesis. The project was successful (Grossman *et al.*, 1984) and Ernest became intrigued and in short order switched the emphasis of his laboratory to AAV. For me it was a great experience, Ernest and Zehava became long-term friends and eventually, Ernest and I had a joint NIH grant for 15 years.

When I first arrived in Gainesville, the dean Al Stetson had said I should be the chair for 6 to 10 years, because it took at least 6 years to accomplish anything and by 10 years whatever I could do would have happened. I also thought it might be nice to live for a while in a world class city. Therefore, when I was offered the chair of Microbiology at

Cornell Medical College in Manhattan shortly after my return from my sabbatical, I decided to accept after gaining Laura's acquiescence (originally, she thought I meant Ithaca and was somewhat taken aback when it became clear I was talking about New York City). For our son, it meant attending the fourth school in 4 years and the third for our daughter. However, they thrived in the Mamaroneck school system and continued to achieve academically and in their chosen careers. For Laura, it gave her the chance to earn her PhD in English Education at NYU.

Cornell worked well; it was on the upper east side in a complex with the Rockefeller University, Memorial Sloan Kettering, New York Hospital, and the Hospital for Special Surgery. I had the chance to interact with all of them. An immediate building process was required in Microbiology; the department had been without a chair for over 8 years and there were only 2.1 faculty FTE in the department. And one of the full time faculty was an ornithologist! Once again there was intensive faculty recruiting with great assistance from Dieter Sussdorf and Larry Senterfit; we initially recruited four people, three of whom are still there, Bill Holloman, Eric Falck-Pedersen, and Francis Barany. Mike O'Donnell eventually moved next door to the Rockefeller as a Hughes investigator. Cornell and Sloan-Kettering more or less collaborated on graduate education. An old friend Bob Krug was the chair of Molecular Biology at Sloan Kettering and I suggested to him that we run a true joint program. He was positive, but did worry whether the two institutions would allow their support funds to be used jointly. In fact, both places were highly supportive and in several years, ours was the largest graduate program in both Cornell and Sloan Kettering. Eventually, there was general fusion of the graduate programs into about 4 areas.

Meanwhile, the work with AAV continued, much of it done by Mark Labow and Ann Beaton on the regulation of AAV gene expression (Labow *et al.*, 1986; Beaton *et al.*, 1989). Chris Leonard purified the AAV rep protein (Leonard and Berns, 1994) and Peter Ward, along with Guang Hong, worked on AAV DNA replication *in vitro* (Hong *et al.*, 1992; Ward *et al.*, 1994; Ward and Berns, 1996). However, the most exciting discovery was a consequence of Rob Kotin's cloning of the cellular sequence flanking the AAV insert in the genome of a human cell line (Detroit 6). Wild-type AAV could be rescued from this cell line by infection with helper adenovirus, even after more than 100 passages. Rob found that the flanking sequence was disrupted in the latently infected cells. When he tested DNAs from independently derived latently infected cell lines provided from the laboratories of Muzyczka and Samulski, we were surprised to find that the same cell sequence had been disrupted and linked to AAV sequences. This result indicated that AAV integrated in a site-specific manner, the first such instance in a mammalian virus. Rob mapped the site to chromosome 19 in collaboration with Marcello Siniscalco and Dave Ward (Kotin *et al.*, 1991). Ernest Winocour visited and worked with postdoc Cathy Giraud to support the target hypothesis (Giraud *et al.*, 1994), which was further analyzed in detail at the nucleotide level by Michael Linden (Linden *et al.*, 1996a, 1996b), Patricio Meneses, and Julie Dyall (Dyall *et al.*, 1999).

In 1995, I served as both the President of the ASM and the chair of the AAMC, which kept me busy and very much involved at the national level with science and medical pol-

icy. Testifying to Congress was stressful, but generally we were received kindly, with the admonition, however, that funds were in short supply. My time with the AAMC got me involved with academic medicine at a level beyond that of the preclinical world. My chair address to the annual AAMC annual meeting was entitled "The Academic Medical Center: Will It Become an Oxymoron?" This is still a problem as the need for patient revenues in clinical departments continues to increase. The notion of enhancing an academic environment throughout a medical school was an interesting challenge. By 1997, I had been a chair at Cornell for 13 years, well beyond the term originally suggested at Florida by Al Stetson, so that when offered a chance to return to Florida as the dean of the College of Medicine, I accepted. David Challoner, the VP for health affairs, made the offer and was my boss. I had known David from earlier at Florida and I knew most of the chairs and many of the senior faculty. Now, instead of being responsible for 15 faculty, it was more like 1500 faculty. More significantly, I was also responsible for the faculty practice, a completely new dimension. Dick Gaintner, an old friend from the National Board of Medical Examiners, was the president of the hospital and I was on the board. This became more critical when after a year David Challoner stepped down as VP and President John Lombardi asked me to assume the role as acting VP, in addition to being the dean. Now I was the nominal chair of the hospital board. Fortunately, Dick was generally cooperative and I had excellent help from my deputy Craig Tisher and from Nick Cassisi, the senior associate dean for clinical affairs. Two major events occurred.

When I became dean, the federal government was conducting an investigation of possible Medicare misconduct, but we managed to resolve the issue at not too great a cost. The second issue was that we were up for an accreditation site visit. Education was the province of Bob Watson and we were well prepared. We got through OK. The high point came during the exit interview of the site visit team with the provost when Dick Krugman, the chair of the site visit, told the provost that he should be proud of having the best medical school in the country at the University of Florida.

We were able to create several bricks and mortar monuments, including several new clinical facilities. The two major building achievements were a proton beam facility on our campus in Jacksonville and a combined cancer-genetics research facility, which was the largest research building on the campus and possibly in the state. The proton beam was suggested by the chair of radiation oncology, Nancy Mendenhall, who said it would cost 20 million. In fact, it cost more than 120 million by the time it was finished, but it has been a great success, a real tribute to Craig Tisher, my deputy and successor, who led the construction effort.

After a couple of years, Chuck Young became president; Chuck had been the president of UCLA for 26 years. We got along and he appointed me the permanent VP. The position kept me fully occupied, but I was still funded to look at AAV. Just before leaving Cornell, Patricio Meneses had starting working with AAV as a vector for gene therapy of the retina to prevent neovascularization (Meneses *et al.*, 2001). I teamed up with Bill Hauswirth and we continued to work on this problem with significant success. Putting an AAV vector carrying a gene for angiostatin or the equivalent into the vitreous humor was successful in preventing retinal

vascularization in the mouse. However, mine was more of a virtual role as Bill was directly in charge.

The experience at Florida was stimulating; as constructed, the VP position did not take up too much of my time, mostly keeping the peace with the hospital and among the six health center deans with central administration. Most of the stimulation derived from being the dean; over my tenure, the level of cooperation from the chairs and almost all the faculty was outstanding. However, in 2002, wanderlust struck and I went back to New York as CEO of Mount Sinai Medical Center. The move was not a success and I returned to Gainesville the next year, this time as the director of the UF Genetics Institute.

In 1997, when I had become the dean, Florida was just entering the public phase of a capital campaign, which was to last until the end of 2000. The overall goal was \$500 million, but the campaign progressed so rapidly that by the summer of 1998 the goal was increased to \$750 million. To justify the increase, some new initiatives were needed. The president, John Lombardi, said since I had kept telling him how important genetics was, UF was going to have a genetics institute, which was to be a university-wide effort and there would be a building for the institute. John left and Chuck Young came in, but the Genetics Institute remained a viable proposal. Although Governor Bush vetoed funding for the new building at least once, eventually, there were sufficient funds to enable construction. Funding was an amalgam of money from a spin-off company, federal funding, state funding, and bonds. The provost, David Colburn, had decided that the building should be a fusion of the buildings proposed for the cancer center and the institute; this was an inspired suggestion, since the investigators in the two were essentially indistinguishable; thus, putting them all under one roof would greatly foster interaction and collaboration. By the time I returned, the building had been designed and I was told construction would commence in 4 months. I altered only one detail, doorways between adjacent labs in the genetics wing remained, but no doors were to be installed, another attempt to foster interaction. To my surprise, construction began almost to the day it had been proposed and the building was occupied in June 2006. It was a great success; that is, there were almost no complaints from the occupants of the building. It was occupied by geneticists from medicine, liberal arts and sciences, and agriculture, the whole idea being to foster novel interaction. Only about 35 genetics faculty could be housed in the new building, but there were more than 230 faculty who were members of the institute. In general, the notion was successful. Interaction was fostered not only by proximity, but also by having some money for collaborative research support, a new graduate program, an active seminar program, and an annual symposium with both external and internal speakers. We were able to recruit at least 10–12 new faculty. In my own laboratory, my final student Shyam Daya graduated in 2009. He had provided evidence that AAV site-specific integration occurred via nonhomologous end joining (Daya and Berns, 2009).

As I look back, I derive satisfaction from having seen AAV progress from being a biological oddity to a leading vector for gene therapy. More satisfying though is seeing the success of the young people who have passed through my laboratory. At the biological level, a primary consideration is

the perpetuation of the genome. At the human level, the young people one has helped to mold represent the equivalent. Similar thoughts apply to the young faculty I helped to recruit as both a chair and dean. I could not say no when asked to participate in various public activities. Although these were frequently time-consuming, I actually took considerable pleasure in having a say in the formation of the national policy regarding science. Today, science is still an attractive goal for excellent students. Although funding is "tight," I feel confident that the best will continue to penetrate the unknown

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