40 Years with Bacteriophage ø29

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Abstract

I have dedicated the past 46 years of my life to science and I expect to be active in research for many more years. I have been lucky in my professional life. During my postdoctoral years I discovered two proteins that I showed to be involved in the initiation of protein synthesis. Working with bacteriophage ø29 for the past 40 years, we have made many interesting findings. Among them is the discovery of a protein covalently linked to the 5′ ends of ø29 DNA that we later showed to be the primer for the initiation of ø29 DNA replication. Also, the finding of the ø29 DNA polymerase with its properties of high processivity, strand displacement, and high fidelity has been very rewarding. The ø29 DNA polymerase has become the ideal enzyme for DNA amplification, both rolling circle and whole-genome amplification. I also am happy because I have worked with many brilliant students and collaborators over the years, most of whom have become excellent scientists.

Contents

CHILDHOOD AND YOUTH

I was born in Canero, Spain, on November 30, 1938. Canero is a small village located in Asturias, on the north coast of Spain, close to Luarca, where the Nobel Prize winner Severo Ochoa was born in 1905.

My father was a physician who specialized in psychiatry and neurology, and my mother, who is still alive, was a school teacher. My parents married in Madrid in 1936, just after the beginning of the Spanish Civil War, and in 1937 they left Madrid and went to Canero, where my grandparents had a country house. They had three children, my brother, José, who is one year older than me, myself, and my sister, María Luisa (Marisa), who is two years my junior. My parents stayed in Canero until the end of the Civil War, in 1939. Then, they moved to Gijón, also located on the north coast of Asturias, where my father set up a psychiatric clinic. My mother, who had to stop her career as a school teacher, managed the clinic. The house where the clinic was established had three floors and one basement. The patients occupied the second and third floors, we lived on the first floor, and the basement was used for general services such as kitchen, laundry, and so on. We liked spending time with the patients and listening to their stories. The house was surrounded by a garden with a tennis court where we enjoyed playing tennis (which I still like but no longer play). As my father was fond of classical music and opera, during the early years of my life I listened to music at home very often, which led to my appreciation of classical music.

When I was four years old I began attending a Catholic school run by nuns, which was usual in Spain during the 1940s. There, I received a good classical education, based in science and humanities, which included Latin and Greek. I studied French and only a few years of English. I received the Baccalaureate title in 1954. My parents were very clear that the three of us should attend university, which was not common for women. My father always told us that the only inheritance he was going to leave us was a university career. That was, indeed, the best legacy he left us. In order to attend the university, it was mandatory to spend one year doing the so-called preuniversity studies. It was then time to choose whether I wanted to follow a scientific or a humanistic career. My decision was clear: I wanted a scientific career. When I finished my preuniversity studies in science, I had to choose from the scientific careers offered a field in which I would specialize. I liked both

medicine and chemistry, the former likely influenced by the fact that my father was a physician. Because I could only study chemistry at the University of Oviedo, which was the university closest to Gijón, I decided, with the agreement of my parents, to attend the Complutense University of Madrid (UCM) to start the first year that was common for both degrees. This allowed me one more year to decide. After I completed my first year I decided to continue to study chemistry. I think that was a good decision because I soon found myself fascinated by the laboratory work that we carried out. During my third year, especially, I spent many hours doing experimental work in organic chemistry, which I liked so much that I thought I would become a researcher in organic chemistry.

FROM ORGANIC CHEMISTRY TO BIOCHEMISTRY

I spent the summer holidays during my years at UCM in Gijón, where my parents lived. In the summer of 1958, when I had finished my third year of chemistry, I was lucky to meet Severo Ochoa. Severo had left Spain in 1936, when the Spanish Civil War began, because he wanted to continue his research. After staying in Germany and England, in 1940 he went to the United States, first to St. Louis, Missouri, to the Washington University School of Medicine, and then to New York, to the New York University Medical Center. For some time he refused to visit Spain, even for vacation, but in 1958 he spent one month of summer in Asturias, half the time in Luarca, his hometown, and the other half in Gijón, where his wife, Carmen, was born. Severo and my father were good friends (they had studied medicine together at UCM, and they had lived at the famous Residencia de Estudiantes, where artists like the painter Salvador Dalí, the film director Buñuel, and the playwright and poet Federico García Lorca had stayed), and they were related by marriage (Severo's uncle, Alvaro de Albornoz, was married to my father's aunt, Amalia Salas).

One day that summer, Severo and Carmen came to visit us and had lunch at our house. I remember we ate an excellent paella. On that occasion I had the privilege of meeting Severo and talking with him about what I was doing and about my thoughts regarding my future. The next day Severo was giving a conference on his work in the nearby city of Oviedo and asked my father and me to accompany him. The three of us went to Oviedo in my father's car. Severo was a brilliant speaker and I became fascinated by his scientific work. I remember he spoke about fatty acid metabolism. At that time I didn't know much about biochemistry because I had not yet studied it at the university; Ochoa promised to send me a biochemistry book. I was excited when one month later I received the book *General Biochemistry*, by Joseph S. Fruton and Sofia Simmonds, a classic, dedicated by Severo Ochoa. When I was about to finish university I had made up my mind to dedicate myself to biochemistry, and I told Severo about my decision. He advised me to do my PhD thesis in Madrid with an excellent biochemist, Alberto Sols, who had trained at the Washington University School of Medicine in St. Louis in the laboratory of Carl and Gerty Cori. Once I finished my PhD thesis, I could go to Severo's lab in New York for postdoctoral training. Ochoa wrote me a reference letter for Alberto Sols, who accepted me right away, even though I was a woman, since he could not refuse something requested by Severo Ochoa, who by 1960 had already won the Nobel Prize. I spent the next four years working on my PhD thesis with Alberto Sols, working on carbohydrate metabolism, specifically on the glucosephosphate isomerase from yeast, which converts glucose-6-phosphate into fructose-6-phosphate. We found that the enzyme has an anomerase-like activity specific for α-glucopyranose-6-phosphate, the intermediate product of which seemed to be the open chain of glucose-6-phosphate. Thus, we had discovered the anomerization of glucose-6-phosphate enzymatically catalyzed by glucosephosphate isomerase. The results

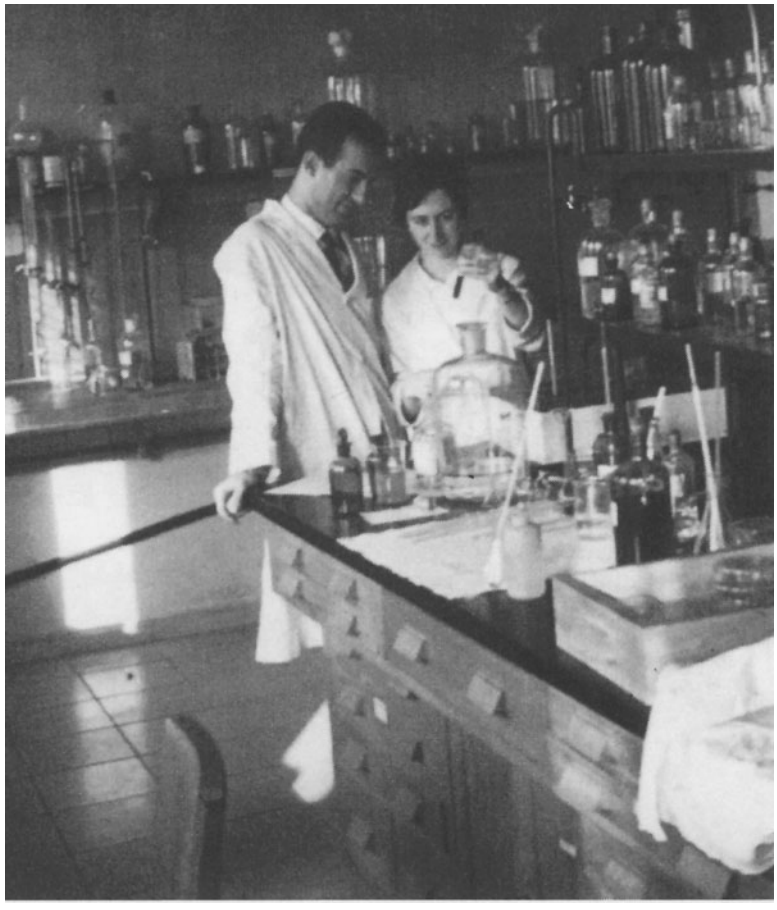

Figure 1

Margarita Salas and Eladio Viñuela at the Center for Biological Research during their PhD thesis work (circa 1962).

of these investigations were published in the prestigious *Journal of Biological Chemistry* (37).

In 1960 I became engaged to Eladio Viñuela, who also began working on his PhD thesis in biochemistry with Alberto Sols (**Figure 1**). Eladio's work dealt with yeast phosphofructokinase, and he demonstrated its allosteric properties. In addition, he discovered a new enzyme in liver, glucokinase, that converts glucose into glucose-6-phosphate, has a high K_m for glucose, 10 mM, and disappears in fasted and alloxan diabetic rats. I joined Eladio in these studies and we later found that glucokinase activity reappears in the diabetic rats after insulin administration and in the fasted rats by refeeding. These results were published in two papers in the *Journal of Biological Chemistry* (36, 47). I emphasize the publications in this journal because at that time it was not easy from Spain to publish in prestigious international journals. For us, this was quite an accomplishment.

While Eladio and I were doing our PhD thesis work, my sister, Marisa, also a chemist, joined Sols' lab and worked with Eladio on the yeast phosphofructokinase (38). In 1962 our father died. My brother, José, who had studied medicine, was doing his military service and he had started his training in neurology. Afterward, he decided to pursue research and also joined Sols' lab to do his PhD thesis. With me, he purified the rabbit liver glucokinase and studied its properties (28). At that time, column chromatography was not used in Sols' lab, as proteins were purified by

precipitation with ammonium sulfate, acetone, or ethanol. I remember that at the end of our stay in Sols' lab, Eladio and I started to use column chromatography for protein purification. Because we did not have a fraction collector, we had to collect the fractions by hand, one by one.

During our PhD thesis work, Eladio and I had modest or even honorary (without stipend) fellowships. In 1963 I received a fellowship from the Spanish Juan March Foundation, and with the money provided we got married and stayed in Madrid one more year to finish our investigations. Because we had the offer from Severo Ochoa to go to his lab at New York University (NYU) for postdoctoral training, we decided to accept and go to New York. Eladio obtained fellowships first from the Juan March Foundation and then from the National Institutes of Health (NIH), and I received fellowships first from the NIH and afterward from the Jane Coffin Childs Memorial Fund for Medical Research. At that time it was a "must" to go abroad for further training, because in Spain the science that could be learned and the possibility of doing research were very limited.

THREE YEARS AT NEW YORK UNIVERSITY

We arrived in New York in August 1964, just in time to attend the International Congress of Biochemistry. I remember the excitement that followed after Phil Leder and Marshall W. Nirenberg's presentation on the use of trinucleotides of specific sequence for the binding of the different aminoacyl-tRNAs. This work led to the final unraveling of the genetic code, showing the specific sequence of trinucleotides coding for the different amino acids, completing the work carried out in the laboratories of Severo Ochoa, Marshall W. Nirenberg, and H. Gobind Khorana.

Once we arrived at Severo Ochoa's lab, he decided that we should work in different groups. He said, "At least, you will learn English, since if you work together you will be speaking Spanish all the time." I think that Ochoa wanted each of us to develop independent research and our own scientific personality. I must say that during the three years we stayed in Ochoa's lab I did not notice any discrimination toward me for being a woman, something that I had suffered in Spain while working on my PhD thesis.

For my research project, Ochoa had me determine the direction of reading of the genetic message, that is, whether the reading was in the 5′ to 3′ or the 3′ to 5′ direction. For the cell-free protein synthesis system we used a supernatant of the bacteria *Lactobacillus arabinosus* that has low nuclease activity and ribosomes from *Escherichia coli* that were washed with 0.5 M NH_4Cl and then applied to a DEAE-cellulose column eluted with 1 M NH_4Cl. We used synthetic template polynucleotides containing the AAC codon at either the 3′ or 5′ end. When we used the polynucleotide 5′ AAAAAA . . . AAAAAC 3′, we found that the amino acids lysine and asparagine were incorporated. Treatment with the enzyme carboxypeptidase A released asparagine but not lysine, indicating that the asparagine was located at the carboxyl end (35). When we used the polynucleotide 5′ AAAAACAAA . . . AAA 3′, the asparagine was incorporated at the amino end as shown because it was not released by treatment with carboxypeptidase A, but with carboxypeptidase B, which hydrolyzes lysines from the carboxyl end (42). I must point out that the triplet AAA at the 5′ end was not translated. Only the AUG triplet at the 5′ end was translated (see below).

After finishing the above project, I started working on the translation of natural mRNA, specifically phage MS2-RNA, using a translation cell-free system obtained from *E. coli*. I used the ribosomes purified as described above and a high-speed supernatant from *E. coli* cell extracts that provided all the soluble proteins and tRNAs. I was surprised to find that, whereas the system was active with poly A as messenger, giving rise to the incorporation of lysine, it was completely inactive

when I used MS2-RNA as messenger. When I precipitated with ammonium sulfate the ribosomal wash, obtained by treating the ribosomes with 0.5 M NH_4Cl, and added this fraction to the purified ribosomes, together with the high-speed supernatant, I recovered the activity with MS2-RNA as messenger. I wondered what the role of the ammonium sulfate fraction was. At that time, Walter (Wally) Gilbert came to NYU to give a seminar about his preliminary results on the isolation of the *lac* repressor, and I showed him my results. He suggested that the factors present in the ribosomal wash could have something to do with the termination of protein synthesis. This was not the case. When I used the synthetic polynucleotide 5′ AUGAAA . . . AAA 3′ as messenger, the results were the same as with MS2-RNA, that is, this synthetic messenger did not work with the washed ribosomes but the activity was recovered when I added the ammonium sulfate fraction. Because the triplet AUG at the 5′ end of a messenger codes for formyl-methionine, this strongly suggested that the factor(s) I was adding could be involved in the initiation of protein synthesis. Indeed, when I used the trinucleotide AUG to study the binding of formyl-methionyl-tRNA to the ribosomes, I found that the two proteins I had purified from the ribosomal wash, which I called F1 and F2 (later called iF1 and iF2), were needed for such binding. This result demonstrated that proteins F1 and F2 were involved in the initiation of translation (31, 34, 43).

By using the cell-free system with the initiation factors and the synthetic polynucleotides 5′AUGUUUAAA . . . AAA3′ and 5′AUGUUUUAAAAA . . . AAA3′ as messengers, we showed that the first polynucleotide produced the synthesis of a polypeptide with the composition formyl-methionyl-phenylalanyl-lysine . . . lysine, whereas the second polynucleotide only gave rise to the incorporation of formyl-methionyl-phenylalanine, indicating that the triplet UAA is a polypeptide chain termination codon (16).

After one year of working with Charles Weissmann on the replication of MS2-RNA, Eladio asked Ochoa if he could develop a project on his own. The project consisted of characterizing the proteins induced in *E. coli* after infection with phage MS2. Ochoa agreed, and Eladio embarked on this project, for which he developed the useful and widely used technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) for the separation of proteins according to their molecular weight (41). We showed that the cell-free system of *E. coli* using MS2-RNA as messenger directed the synthesis of two of the three proteins synthesized in vivo after infection of *E. coli* with phage MS2 (45). The two in vitro–synthesized proteins contained formyl-methionine at the amino end, supporting the view that formyl-methionine is involved in the initiation of each of the polypeptide chains programmed by the polycistronic messenger RNAs of *E. coli* phages (46).

I have unforgettable memories of my stay in Severo Ochoa's lab. He taught us (Eladio and me) not only molecular biology, which we would later develop and teach in Spain, but also his experimental rigor, his dedication to and enthusiasm for research. He followed thoroughly the work we carried out, and we had daily discussions with him regarding the experiments we had done and the ones we planned to do. Particularly pleasant was lunchtime, when, in addition to long discussions about science, we would also talk about music, art, literature, and travels. Everyday at noon sharp Severo Ochoa picked us up from our labs to go to the faculty dining room to have lunch. I have also good memories of the classes given to the medicine students by the professors of the department. All members of the department attended these classes. This gave us the opportunity to learn molecular biology from the theoretical viewpoint of Severo Ochoa and other excellent professors of the department.

I would like to mention some of the scientists from the biochemistry department who

at the time were leading their own research. Bob Warner, an excellent physical chemist and highly appreciated by Ochoa, characterized, among other things, the polymer formed after mixing poly A and poly U synthesized by the polynucleotide phosphorylase discovered by Marianne Grunberg-Manago and Ochoa in 1955 (12). Bob Chambers, an organic chemist who had been a postdoctoral student of Gobind Khorana, had an excellent background in nucleic acid chemistry. He made important contributions to the biochemistry of tRNA by trying to define the structural determinants recognized by aminoacyl-tRNA ligases. Charles Weissmann, a brilliant scientist, worked on the replication mechanism of RNA phages as MS2 and Qβ. Albrecht Kleinschmidt, a famous electron microscopist, pioneered the technique of visualizing DNA and protein with the electron microscope. His photograph of the DNA released from the head of a T2 phage by osmotic shock is, according to Ochoa, one of the most impressive in molecular biology. M. Daniel Lane, an excellent scientist and expert on fatty acid synthesis, was the person with whom Eladio and I identified the most. We became very good friends with him and his wife, Pat.

BACK TO SPAIN: PHAGE ø29 AS A MODEL SYSTEM

After three years in Severo Ochoa's lab, Eladio and I decided to return to Spain to try to develop the molecular biology that we had learned with Ochoa. Although we knew it would be difficult to conduct high-level science in Spain, we wanted to try. We had to decide what project we wanted to work on. It was clear to us that we should not continue the same line of research carried out in Ochoa's lab. The previous summer (1966) we had followed the Bacteriophage Course at Cold Spring Harbor, where we learned how to work with phages (**Figure 2**). Thus, we decided to choose a phage as a model system and to study it at the molecular level, including the morphogenesis of the phage particle, as well as the basic processes of genetic information transfer such as replication and transcription. We would work with a lesser-known phage so that there would be less competition, because we knew there would be a delay before we could get results from our work in Spain. Eladio and I decided to work together because it would be difficult to start a research group in Spain, and we thought it would be easier if we joined and complemented our efforts.

Figure 2

Margarita Salas (bottom row, fourth from left) and Eladio Viñuela (directly behind her) at the Bacteriophage Course (1966) at Cold Spring Harbor Laboratories.

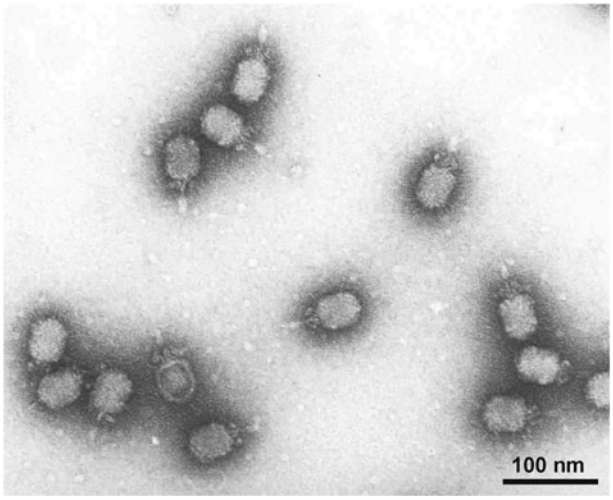

Figure 3

Electron micrograph of bacteriophage ø29.

After much reading and thinking, Eladio found a paper published by Dwight L. Anderson's lab describing the morphology of the particle and the size of the DNA of a *Bacillus subtilis* phage (1). The name of the phage, initially characterized by Bernie Reilly in John Spizizen's lab, was ø29, and it had the characteristics we were looking for: it was small in size, it had a rather complex morphology (see **Figure 3**), and very little was known about it. Therefore, we wrote a grant proposal for the Jane Coffin Childs Memorial Fund for Medical Research, proposing that we would use phage ø29 as a model system for morphogenesis and to study the mechanisms of genetic information transfer. Thanks to this grant, undoubtedly obtained with the help and support of Severo Ochoa, we were able to begin our work, because at that time (1967) financial assistance for research was not available in Spain. After learning that we had obtained the grant and having with us phage ø29, obtained from Dwight Anderson, we returned to Madrid in July 1967 to embark on our scientific adventure.

EARLY YEARS IN MADRID

During our stay in New York Eladio and I were appointed as scientific collaborators of the Spanish National Research Council (CSIC). We started our research at the Center for Biological Research, where some years before we had carried out our PhD thesis work. The director of one of the institutes at the Center, José Luis Rodríguez Candela, offered us a large lab. Of course, the lab was empty and, with the funds we obtained from the Jane Coffin Childs Grant, we bought some small equipment and the reagents needed to start our project. Times were difficult in Spain, and one of the first things we had to know was how to get the products and equipment. When we started the lab in September 1967, Eladio and I were alone. Generously, the Center provided us with a technician. I remember our first experiment: to grow a culture of the bacteria *Bacillus amyloliquefaciens*, which was a better host for ø29 than *B. subtilis*; to infect the culture with the phage; and to see whether, after 40–50 min at 37°C, the bacteria was lysed. We were excited to see the bacteria lysing because that meant we had obtained the system needed to go on with the project. As I mentioned before, the only characteristics known about the phage was the size of the DNA (a molecular mass of about 12 million Da) and its morphology from an electron micrograph, published by Dwight Anderson's lab (1). Therefore, we decided to start from the beginning: to look at the structural proteins of the phage, do the genetics by isolation of conditional lethal mutants, isolate the phage DNA, and study its transcription. Fortunately, a few months after our arrival in Spain, the first predoctoral fellowships were awarded and we could start looking for our first students.

The first student was Enrique Méndez, who characterized the structural proteins of the phage particle by using the technique of SDS-polyacrylamide gel electrophoresis developed by Eladio at NYU. Later, Galo Ramírez, who we knew through a mutual friend, and who was working in neurology, was convinced by Eladio to learn molecular biology techniques and joined the lab to work on the head-neck connecting protein in ø29. After Enrique, Jesús Ávila and Antonio Talavera arrived at our lab. Jesús was engaged

in the study of the *B. subtilis* RNA polymerase, which would be used for the transcription of the phage DNA. He showed that, as in the case of the *E. coli* RNA polymerase, the *B. subtilis* enzyme was composed of several subunits corresponding to the *E. coli* β, β′, σ, and α. José M. Hermoso, who arrived later, joined this project. We were very proud when the first paper describing these results was published in *Nature* (2). I was also excited when I received a letter from Jim Watson to attend the Cold Spring Harbor Symposium on transcription. There, I found that Richard Losick had obtained results similar to ours.

We gave to Antonio Talavera the project of isolating conditional lethal mutants of ø29. We wanted to isolate both temperature-sensitive (*ts*) and suppressor-sensitive (*sus*) mutants, but we did not know whether the *Bacillus* strain we were using was suppressor negative (su^-) or suppressor positive (su^+). Therefore, we decided to isolate *ts* mutants. Antonio did a good job and he soon had a collection of *ts* mutants that he mapped, and he also characterized the mutants that were affected in ø29 DNA synthesis. A few years later, Felipe Moreno, a Spanish scientist working in Paris, asked to work on the ø29 project for his PhD thesis. We gave him a risky task: isolate *sus* mutants of ø29. He used as su^- bacteria *B. subtilis* 110NA (a strain spoOA$^-$ in which ø29 grows readily), and *B. subtilis* 168 MO99 spoOA$^-$ su^{+3} as su^+. Felipe succeeded in isolating ø29 *sus* mutants; Ana Camacho characterized that the *B. subtilis* strain used was a weak suppressor of nonsense mutations, and then Rafael P. Mellado isolated a strong suppressor of nonsense mutations in *B. subtilis*. In parallel to our work, Bernie Reilly in Dwight Anderson's lab had also isolated a collection of ø29 *ts* and *sus* mutants, and we decided to combine the two collections in a genetic map in which 17 genes were characterized and a linear genetic map of 24.4 recombination units was constructed, with the genes numbered sequentially from left to right (1 to 17) according to their relative map position (18). Genes 1–6 and 17 were early genes, whereas genes 7–16 were late genes. A physical map relative to the genetic map was also constructed by Marta R. Inciarte and José M. Lázaro using marker rescue experiments with the restriction nuclease EcoRI. Restriction nucleases were not available at that time, and José M. Lázaro had to purify the EcoRI enzyme. This was the first time that a restriction enzyme was used in Spain. Meanwhile, José L. Carrascosa characterized the proteins induced in *B. subtilis* after infection with phage ø29. This was not an easy task because ø29 infection does not stop bacterial protein synthesis. Therefore, only the more abundant proteins could be characterized.

Using the available *sus* mutants, Ana Camacho, Fernando Jiménez, José L. Carrascosa, and Javier de la Torre characterized the morphogenetic route for the assembly of the proteins and the encapsidation of DNA to make a ø29 phage particle. We came up with results similar to those obtained in Dwight Anderson's lab.

Soon after we started our work in Madrid, Juan Ortín and Víctor Rubio joined our group. Víctor studied the biophysical properties of the phage, and Juan isolated and characterized the phage DNA. We were surprised to see that the phage DNA was not isolated in a linear form, as it was supposed to be, but as circular molecules and concatemers, as it was visualized with the electron microscope by Cesar Vásquez, an Argentinean scientist who had trained at NYU with Albrecht Kleinschmidt and had joined our lab for a few months. Our surprise was even greater when we found that the circles and concatemers were converted into unit-length ø29 DNA when treated with a proteolytic enzyme such as trypsin. This meant that, somehow, protein was involved in the circularization and concatemerization of ø29 DNA. To our delight, this paper was published in *Nature New Biology* (22). Two years after our publication, Robinson and coworkers published a similar result with adenovirus DNA (25).

Later, Rekosh and coworkers (24) characterized a protein covalently linked at the

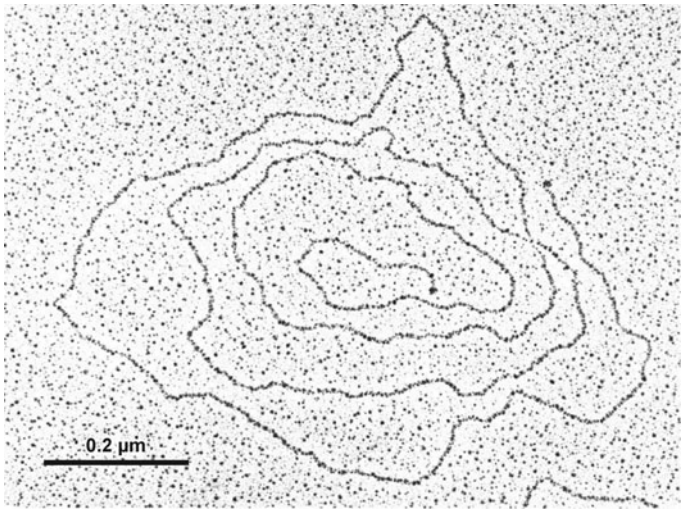

Figure 4

Electron micrograph of the protein-DNA complex of bacteriophage ø29 (photo taken by José M. Sogo).

5′ ends of adenovirus DNA and proposed a model in which a free molecule of the protein could act as a primer for the initiation of replication by forming a covalent linkage with dCMP, the 5′-terminal nucleotide, which would provide the 3′-OH group needed for elongation by the DNA polymerase. We also characterized a protein covalently linked to the 5′ termini of phage ø29 DNA (32) that we later showed was involved in the initiation of ø29 DNA replication. This protein was called terminal protein (TP). **Figure 4** is an electron micrograph taken by José M. Sogo showing a linear ø29 DNA molecule with the TP at the DNA ends. Viewing the protein with an electron microscope was not easy because the TP is 31 kDa.

As I mentioned above, we had characterized the *B. subtilis* RNA polymerase, and earlier studies by José M. Hermoso and later studies by José M. Sogo, Marta R. Inciarte, and Javier Corral determined the existence of early and late transcription in ø29 DNA, mapping the ø29 promoters and showing that after infection with a *sus*4 mutant late transcription was impaired, suggesting that the gene 4 product was involved in ø29 late transcription. There were several possibilities: (*a*) the gene 4 product could be a new RNA polymerase that recognizes the late promoter(s); (*b*) it could be a sigma factor that replaces the σ^A factor of *B. subtilis* RNA polymerase; or (*c*) it could be an activator that helps the *B. subtilis* RNA polymerase to transcribe the late promoter(s). There was a report suggesting that the gene 4 product could be a new RNA polymerase, and Fernando Jiménez tried to show this without success. At that time (1973) there was a gas explosion at the Center of Biological Research, and for a few months we had to leave our experiments. It was the month of June and the members of our group met everyday in a nearby Center, the Instituto Rocasolano, for seminars and scientific discussions. When we returned to the lab we continued to try to find out how the gene 4 product controlled the switch from early to late transcription. One challenge was trying to characterize the protein product of gene 4, which was difficult if the protein was not synthesized in large amounts, because ø29 infection does not stop host protein synthesis. We were lucky because the tools of genetic engineering had become available, and we could clone genes and overproduce proteins. This changed the ø29 work and led to new possibilities for us.

Another change took place in the ø29 group in the early 1970s. Eladio decided to start a new project, the study of the molecular biology of African Swine Fever virus, which was a plague in Extremadura, his homeland. This was a generous move on his part because it allowed me to lead the ø29 work and show my colleagues whether I was able to develop the research on my own, which was important for me at a time and in a country where the scientific work of a woman was of little value. Eladio established a powerful group that became a leader in the field. I was lucky. I worked hard and had good students, and Eladio helped me continuously. I think I was successful working on ø29 and I became a scientist in my own right.

I would also like to mention that every four years, from 1980 to 1996, I organized in Salamanca, Spain, an International

Workshop on Bacteriophages funded by EMBO. This attracted the best phage workers from both Europe and the United States that were acquainted with the kind of science that was being done in Spain.

In 1977 we moved to the new Center of Molecular Biology "Severo Ochoa," which was built with the idea of bringing Severo Ochoa back to Spain. The scientific contributions of Eladio and the technical contributions of Javier Corral and Juan A. Manzanares made possible the existence and quality of the Center.

The work carried out since our move to the Center of Molecular Biology is mainly centered on the study of the mechanisms of control of ø29 DNA transcription and of TP-primed ø29 DNA replication. But before I continue the description of the work, I would like to mention the good times had by the ø29 and African Swine Fever virus groups. Every summer in July we used to meet at our country house. We played tennis, but, above all, we enjoyed having ping-pong matches. Both groups formed competitive teams, which had very good players, and the games were well-matched.

CONTROL OF ø29 DNA TRANSCRIPTION

At the beginning of ø29 infection only the genes involved in DNA replication and transcription regulation are expressed. Genes coding for the structural proteins of the phage particle and for proteins involved in morphogenesis and cell lysis are expressed later in infection. After the work of José M. Sogo, who had initially mapped σ^A-RNA polymerase binding sites by electron microscopy, Isabel Barthelemy and Rafael P. Mellado localized transcription initiation sites by S1 mapping and identified the sequences of the four main early promoters, A1, A2c, A2b, and C2, and of the late promoter A3. The early promoters have –10 and –35 hexamers and are recognized by the σ^A-RNA polymerase, whereas the late promoter, which lacks a –35 hexamer, requires the product of gene 4, in addition to the σ^A-RNA polymerase. The weak early promoter A1, which is located at the left end of the genome, gives rise to a small transcript that participates in the packaging of DNA into proheads (13). The A2c and A2b promoters are responsible for the expression of genes 6, 5, 4, 3, 2, and 1, as well as that of a new gene, called 56 because it encodes a protein of 56 amino acids. These genes are involved in DNA replication (6, 5, 3, 2, 1, and 56) and control of transcription (4 and 6). The C2 promoter, located at the right end of the genome, drives the expression of gene 17 and the more recently discovered gene 16.7, both of which are involved in DNA replication, and is repressed by protein p6. C2 is transcribed first because, as shown by Víctor González-Huíci and José M. Hermoso, the 65% right end DNA is injected first into the bacteria, pushed into the cell likely because of the pressure built up inside the viral capsid. In agreement with the right-to-left polarity for DNA entry into the infected cell, Steve Kraviec had shown earlier that ø29 DNA is released in vitro from the phage particles with a right-to-left polarity. Previous to injection, the phage adsorbs specifically to the bacterial cell wall through the neck appendages of the viral particle as shown by Nieves Villanueva (44). The late A3 promoter, located close to the A2b promoter and transcribed in the opposite direction, is responsible for the expression of genes 7 to 16, encoding structural, morphogenetic, and lysis proteins.

The characterization of the gene 4 product was made possible owing to two new developments: sequencing of the gene, done by Cristina Escarmís, and cloning of the gene in *E. coli* under the control of the P_L promoter of phage λ, done by Rafael P. Mellado, for overproduction of the protein. Purification of p4 and the setup of an in vitro transcription assay by Isabel Barthelemy allowed us to characterize p4 as a transcriptional activator of the late A3 promoter. Transcription from A3 required not only p4 but also the σ^A factor of RNA polymerase, ruling out the possibility that p4

was an alternative sigma factor. DNase I footprinting assays showed that the binding site for p4 was localized at a region spanning positions –56 to –102 relative to the transcription start site of the A3 promoter; this region was shown to have static curvature. Circular permutation assays showed that p4 increases the curvature of its binding site from about 45° to 85° without changing the bend center. As shown by Beatriz Nuez, promoter A3 activation implies the p4-mediated stabilization of RNA polymerase at the promoter as a closed complex. The binding site for p4 at the late promoter overlaps with the –35 hexamer at the oppositely oriented early A2b promoter, so that p4 and RNA polymerase cannot bind simultaneously to this site. As a result, protein p4 excludes RNA polymerase from promoter A2b, repressing it, as shown by Fernando Rojo (27). The early A2c promoter, located 96 bp downstream from A2b, is also repressed by protein p4 through a mechanism that implies the simultaneous binding of p4 and RNA polymerase to the promoter, preventing the escape of the polymerase from it, as shown by María Monsalve, Mario Mencía, and Fernando Rojo. Interestingly, both promoter A3 activation and A2c repression require interaction between the amino acid Arg120 of p4 and the carboxy-terminal domain of the α subunit of *B. subtilis* RNA polymerase (27). We further showed that transcription activation or repression by protein p4 depends on the strength of the RNA polymerase-promoter interactions. Thus, when the –35 hexamer was removed from the A2c promoter, protein p4 activated the promoter instead of repressing it, whereas inclusion of a –35 hexamer at the A3 promoter led to its repression by p4 (21). Meanwhile José A. Hocajadas studied the switch from early to late transcription in the ø29-related phage GA-1; this switch is also dependent on the regulatory protein $p4_G$.

Montserrat Elías-Arnanz later showed that expression of the A2b, A2c, and A3 promoters is regulated by the ø29 protein p6 as well as by p4 (11). Protein p6 is a nucleoid-type protein highly expressed in ø29-infected cells that is involved in the initiation of ø29 DNA replication (see below) and also inhibits the early C2 promoter (as indicated above). In addition, protein p6 promotes p4-mediated repression of the A2b promoter and activation of the A3 promoter by enhancing binding of p4 to its recognition site at promoter A3; on the other hand, p4 promotes p6-mediated repression of the A2c promoter by favoring the formation of a stable p6-nucleoprotein complex that interferes with RNA polymerase binding to it. Using hydroxyl radical footprinting Ana Camacho demonstrated that p4 binds to four sites: sites 1 and 2 located between promoters A2c and A2b, and sites 3 and 4 between promoters A2b and A3. In the nucleoprotein complex formed by the cooperative binding of proteins p4 and p6, two dimers of p4 bind to sites 1 and 3, and three dimers of p6 bind between them. As shown by Belén Calles, the stable assembly of this complex requires interaction between protein p6 and the C terminus of protein p4.

The crystal structure of p4 alone and in complex with a 41-bp DNA, including the A3 promoter binding site with the target sequence 5′-AACTTTTT-15bp-AAAATGTT-3′, has recently been determined by Daniel Badía and Miquel Coll in a collaboration established between Coll's lab and mine. Protein p4 has a unique α/β fold that includes a new DNA recognition motif consisting of two N-terminal β turn substructures, or N-hooks, located at the tips of an elongated protein homodimer. The two N-hooks, one of each monomer, enter the major groove of the double helix, establishing base-specific contacts. A high DNA curvature allows p4 N-hooks to reach two major groove areas three helical turns apart. The relevance of the different residues for DNA binding was assessed by Laura Pérez-Lago, Cristina Escandón, and Ana Camacho, who prepared site-directed mutants and tested their binding to DNA by band-shift assays. Mutants Thr4Ala and Arg6Ala, both at the N-hooks, as well as Tyr33Ala, had severely reduced their ability to interact with site 3 (3). The results

indicated that the only base-specific contacts are between Arg6 and the G residues at the inverted repeat of the target sequence; the rest of the contacts are with the phosphate backbone. More recently, Ana Camacho showed that, in addition to the Arg6-G-specific contacts, there is DNA sequence-specific recognition through indirect readout of A-tracts.

PROTEIN-PRIMED ø29 DNA REPLICATION

The sequence at the ends of ø29 DNA, as well as that of gene 3, coding for the TP was determined by Cristina Escarmís. The ends of ø29 DNA contain an inverted terminal repeat six nucleotides long (AAAGTA). The TP, 266 amino acids long, is linked to the DNA ends by a phosphoester bond, which is sensitive to alkali, between the OH group of serine residue 232 and 5′ dAMP, as determined by José M. Hermoso and Enrique Méndez.

Electron microscopic analysis of the replicative intermediates synthesized in *B. subtilis* infected with ø29, carried out by José M. Sogo and Marta R. Inciarte, showed the presence of two basic types of replicating molecules similar to those found in adenovirus-infected cells. Type I molecules consist of double-stranded DNA with single-stranded tails coming from one or two DNA ends; type II molecules are partially double stranded and partially single stranded. Analysis of these molecules showed that replication starts at either DNA end, nonsimultaneously, and proceeds toward the other end by strand displacement.

When Miguel A. Peñalva incubated extracts from ø29-infected *B. subtilis* with [α-^{32}P]dATP in the presence of Mg^{2+} and ø29 TP-DNA as template, a ^{32}P-labeled protein with the electrophoretic mobility of the TP was found. Incubation of the ^{32}P-labeled protein with piperidine released 5′ dAMP, indicating the formation of a TP-dAMP covalent complex (23).

Shift-up experiments in *B. subtilis* infected with *ts* mutants in gene 2 or 3 showed that these two genes are involved in the initiation of ø29 DNA replication. Later, Luis Blanco, Juan A. García, and Miguel A. Peñalva showed that both genes are essential for the in vitro initiation reaction, that is, for the formation of the TP-dAMP covalent complex. Genes 2 and 3 were cloned by Luis Blanco and Juan A. García, respectively, under the control of the P_L promoter of phage λ, and both proteins were overproduced and purified by Luis Blanco (p2) and Ignacio Prieto and José M. Lázaro (p3). In addition to catalyzing the initiation reaction, the purified protein p2 was shown by Luis Blanco to have DNA polymerase activity when assayed with a template primer. The ø29 DNA polymerase has 3′–5′ exonuclease activity on single-stranded DNA and has properties expected for an enzyme involved in proofreading, as shown by Luis Blanco, Cristina Garmendia, and José A. Esteban.

Luis Blanco also showed that the purified system consisting of TP and DNA polymerase with ø29 TP-DNA as template gave rise to the synthesis of a high level of full-length ø29 DNA in a very processive way (5, 7). When primed M13-DNA was used as template, the ø29 DNA polymerase synthesized DNA chains greater than 70 kb (5), indicating that the ø29 DNA polymerase produces strand displacement without the need for accessory proteins. As a result of these two properties, processivity and strand displacement capacity, as well as its proofreading activity, ø29 DNA polymerase (30) has been developed commercially as a tool for isothermal rolling-circle amplification (10) and whole-genome amplification (9).

A Sliding-Back Mechanism to Initiate TP-Primed DNA Replication

Juan A. García and Julio Gutiérrez showed that TP-free ø29 DNA fragments are active templates in vitro for the protein-primed initiation and elongation of replication, although their activity is 10-fold lower than that of TP-DNA. Similar results were obtained with

single-stranded oligonucleotides, with the sequence corresponding to the 3′ terminus of ø29 DNA. Luis Blanco had shown that, in the absence of DNA, ø29 DNA polymerase could add any of the four dNTPs to the TP. Moreover, the presence of the DNA specifically stimulated the formation of the TP-dAMP complex, implying that the TP-primed initiation reaction was a templated event. This finding led Juan Méndez to test single-stranded oligonucleotides with changes in the first, second, or third position relative to the wild-type 3′-terminal sequence. To our amazement, he showed that initiation does not take place at the 3′-terminal nucleotide but at the second position from the 3′ end. The DNA ends are recovered by a specific mechanism that we called sliding-back. Once the TP-dAMP initiation complex has been formed, directed by the second nucleotide (T) at the 3′ end, the TP-dAMP complex slides backward, locating the dAMP in front of the first nucleotide of the template (asymmetric translocation). Then, the next nucleotide (A) is incorporated into the TP-dAMP initiation complex, again using the second T of the template as a director (19). Internal initiation also occurs in other ø29-related phages such as Nf and GA-1, as shown by Elisa Longás and Belén Illana; in the *Streptococcus pneumoniae* phage Cp-1, as shown by Ana Martín in a collaboration between my lab and Pedro García's lab; in the *E. coli* phage PRD1, as determined by Javier Caldentey in a collaboration between Dennis Bamford's lab and mine; and in adenovirus, as shown by Peter van der Vliet's lab (reviewed in Reference 33). To initiate ø29 DNA replication, the viral DNA polymerase must interact with a free molecule of the TP to prime DNA synthesis at each ø29 DNA end. Juan Méndez and Luis Blanco showed that the DNA polymerase-primer TP heterodimer is not dissociated immediately after initiation. There is a transition stage in which the DNA polymerase synthesizes a five-nucleotide-long DNA molecule while complexed with the primer TP; then, it undergoes some structural change during replication of nucleotides 6–9, and finally dissociates from the primer protein when nucleotide 10 is incorporated into the nascent DNA chain (20).

Structural-Functional Studies on the ø29 DNA Polymerase and Terminal Protein

When this part of the work was started, the three-dimensional structure of ø29 DNA polymerase was not available. Therefore, Luis Blanco and Antonio Bernad compared the amino acid sequences of prokaryotic and eukaryotic DNA-dependent DNA polymerases and found a number of conserved motifs at both the N- and C-terminal domains that served to identify novel DNA polymerases in TP-containing linear plasmids. We proposed that the 3′–5′ exonuclease active site of prokaryotic and eukaryotic DNA polymerases is evolutionarily conserved, formed by three N-terminal amino acid motifs (ExoI, ExoII, and ExoIII) invariantly containing four carboxylate groups that bind two metal ions and one tyrosine residue that is involved in orienting the attacking water molecule (4). José A. Esteban performed a steady-state analysis on mutants lacking the carboxylic groups of residues Asp12 and Glu14 (ExoI motif), Asp66 (ExoII motif), or Asp169 (ExoIII motif) that indicated that the mutations led to a 100-fold reduction of the exonuclease activity. Further analyses of other residues were conducted by Miguel de Vega, who showed that amino acids such as Asn62 and Phe65, among others, are involved in single-stranded DNA binding. Site-directed mutagenesis of conserved amino acids at motifs DX_2SLYP, KX_3NSXYG, TX_2GR, YXDTDS, and KXY in the C-terminal domain of ø29 DNA polymerase showed that this domain is involved in both polymerization and protein-primed initiation, and identified amino acids involved in metal binding and catalysis, DNA binding, TP binding, and dNTP interaction (for a review see References 8, 29).

Many other predoctoral and postdoctoral students, in addition to the ones already

mentioned, have contributed to this work. I mention them in chronological order: María A. Blasco, Eulalia Parés, Juan Méndez, Marisol Soengas, Javier Saturno, Verónica Truniger, Tatiana Ilyina, Francisco Esteban, Ana Bonnin, Emmanuelle Dufour, Ralf Eisenbrandt, Gonzalo Fernández-Miranda, Irene Rodríguez, and Patricia Pérez-Arnáiz. In addition, I would like to mention José M. Lázaro, who is the lab expert on protein purification and did the tremendous job of purifying the high number of DNA polymerase mutants that have been used throughout this work. I would also like to mention Laurentino Villar, who efficiently helps us with protein purification.

Recently, the crystal structure of ø29 DNA polymerase has been determined through a collaboration between my and Tom Steitz's labs. The structure of ø29 DNA polymerase provides explanations for its extraordinary processivity and strand displacement activities. Homology modeling suggests that template DNA passes through a tunnel prior to entering the polymerase active site. This tunnel is too small to accommodate double-stranded DNA and requires the separation of template and nontemplate strands. DNA polymerases that use a protein as primer contain two sequence insertions, which we named TPR1 and TPR2. TPR1 forms a domain not previously observed in polymerases, whereas TPR2 resembles the specificity loop of T7 RNA polymerase. The high processivity of ø29 DNA polymerase may be explained by its topological encirclement of both the template strand and upstream duplex DNA (14).

To analyze the functional role of the TPR2 insertion, Irene Rodríguez and Miguel de Vega constructed a ø29 DNA polymerase deletion mutant lacking TPR2 amino acid residues Asp398 to Glu420. Analysis of the mutant DNA polymerase showed that its DNA binding capacity is diminished, drastically decreasing its processivity. In addition, the intrinsic capacity of ø29 DNA polymerase to perform strand displacement coupled to DNA synthesis was abolished in the deletion mutant. Thus, these results demonstrated that the TPR2 insertion plays a critical role in strand displacement and processivity (26).

The specificity of Ser232 in the TP was determined by Cristina Garmendia, who showed that a change of this amino acid into threonine completely abolished the primer activity. On the other hand, deletion and site-directed mutants of the TP constructed by Ángel Zaballos pointed to amino acids required for interaction with the DNA polymerase.

The crystal structure of the ø29 DNA polymerase: terminal protein heterodimer, obtained through a collaboration with Tom Steitz' lab, shows one domain of TP (N-terminal) making no interactions, a second domain (intermediate) binding the polymerase, and a third domain containing the priming serine residue occupying the same binding cleft in the polymerase as duplex DNA does during elongation. Thus, the progressively elongating DNA duplex product must displace this priming domain. In addition, the heterodimer of polymerase and TP cannot accommodate upstream DNA, explaining its specificity for initiating DNA synthesis only at the ends of the phage genome. A model was proposed for the transition from the initiation to the elongation phases in which the priming domain of TP moves out of the active site as polymerase elongates the primer strand. According to this model, the TP should dissociate from polymerase after the incorporation of approximately six nucleotides (15), which is in good agreement with biochemical data that indicate dissociation occurs after 6–10 bases have been incorporated (20).

Viral Proteins p6 and p5 Essential for ø29 DNA Replication

The double-stranded DNA binding protein p6 is a histone-like protein essential for in vivo ø29 DNA replication and one of the most abundant proteins in ø29-infected

B. subtilis, amounting to about 700,000 molecules per cell at late infection times, as shown by Ana Abril. Ricardo Pastrana first cloned gene 6 and overproduced the protein showing that protein p6 is a dimer of 24 kDa. Binding of p6 to the ø29 DNA ends stimulates the formation of the TP-dAMP initiation complex by decreasing the K_m for dATP and facilitating the transition from initiation to elongation. As shown by Ignacio Prieto and Manuel Serrano, protein p6 binds as a dimer to ø29 DNA, preferentially to the DNA ends, every 24 nucleotides in a cooperative way, forming a peculiar nucleoprotein complex. Manuel Serrano showed that p6 binding to circular DNA restrains positive supercoiling, supporting a model in which a right-handed DNA superhelix tightly wraps around a multimeric p6 core (39). Using electron microscopy, Crisanto Gutiérrez showed that the DNA in the complex with p6 is compacted 4.2-fold. The parameters that define the path followed by the DNA in the p6 complex have been determined, indicating that the DNA would be slightly bent (66° every 12 bp) and underwound (11.5 bp per turn). These features would facilitate the initial unwinding of DNA required to start replication.

By deletion and site-directed mutagenesis M. Jesús Otero and Raimundo Freire showed that the N-terminal region of p6 is involved in DNA binding; specifically, mutations at amino acids Lys2 and Arg6 impaired DNA binding that occurs through the DNA minor groove. Ana Abril identified residues critical for p6 dimerization (Ile8 and Ala44) that are also impaired in DNA binding and in the initiation of ø29 DNA replication. Thus, dimers seem to be the active form of p6 for DNA binding. By combining heterologous components from ø29-related phages such as Nf and GA-1, we showed that the activation of replication origins requires the specific recognition of initiation proteins to nucleoprotein complexes. Binding of protein p6 to ø29 DNA has been used by Víctor González-Huíci, Martín Alcorlo, and José M. Hermoso to show the topological restriction of ø29 DNA in the infected cell, as well as to study ø29 DNA ejection into *B. subtilis*.

Gene 5 of ø29 encodes the single-stranded DNA binding protein p5 (SSB), which is abundant in ø29-infected *B. subtilis* and essential for elongation of replication in vivo. Using electron microscopy, Crisanto Gutiérrez showed in vitro binding of ø29 SSB to ø29 DNA replicative intermediates. As shown by Gil Martín, binding of the SSB to ø29 DNA stimulates dNTP incorporation and increases the elongation rate, mainly when ø29 DNA polymerase mutants impaired in strand displacement are used. This likely occurs because of the helix-destabilizing activity of the ø29 SSB, as shown by Marisol Soengas. The presence of the SSB is critical for preventing degradation of the single-stranded DNA and for avoiding the formation of subgenomic DNA molecules (see below). Protein p5 binds single-stranded DNA in a cooperative way, covering 3.4 nucleotides per monomer. A comparative study of the structural complexes formed by the ø29, Nf, and GA-1 SSBs, carried out by Irene Gascón, indicated that the binding site size of Nf SSB is similar to that of ø29 SSB (4.7 nucleotides per monomer), whereas GA-1 SSB, which behaves as a hexamer, covers 51 nucleotides. In addition, the length of circular single-stranded DNA was reduced sixfold upon GA-1 SSB binding and only twofold when ø29 SSB was bound. In agreement with the structural data, less GA-1 SSB than ø29 or Nf SSB was needed to stimulate in vitro DNA replication or for a similar helix-destabilizing activity. An insertion of 40 amino acids is present in GA-1 SSB compared with ø29 or Nf SSB. Deletion analysis showed that the region comprising amino acids 19 to 26 is essential for GA-1 SSB oligomerization.

In Vitro Amplification of ø29 DNA

By using the four purified ø29 replication proteins described above—TP, DNA polymerase, protein p6, and protein p5 (SSB)—Luis Blanco amplified in vitro small amounts (0.5 ng) of the 19,285-bp-long ø29 DNA by

three orders of magnitude (0.5 μg) after 1 h of incubation at 30°C. The quality of the amplified DNA was demonstrated by transfection experiments, in which the infectivity of the amplified DNA, measured as the ability to produce phage particles, was identical to that of the natural ø29 DNA obtained from virions (6). José A. Esteban showed that, in the absence of protein p5, aberrant ø29 DNA replication occurs and subgenomic DNA molecules (named invertrons) that have identical origins at both DNA ends are formed, originated by a snap-back mechanism.

Membrane Proteins Involved in ø29 DNA Replication: Proteins p1 and p16.7

Phage ø29 DNA replication was strongly reduced when nonsuppressor *B. subtilis* cells were infected with a *sus*1 mutant phage. As shown by Alicia Bravo, protein p1 associates with the cell membrane and the 43 C-terminal amino acids are required for this association. In addition, protein p1 lacking the 33 N-terminal amino acids assembled into long protofilaments associated in a highly ordered, parallel array, forming two-dimensional sheets. Gemma Serrano-Heras demonstrated in vivo that p1 self-associates, and large multimeric structures are associated to the bacterial membrane. Protein p1 also interacts with the TP in vitro. Altogether, the results led us to suggest that p1 is a component of a virus-associated membrane structure that provides an anchoring site for the phage DNA replication machinery.

Gene 16.7, located at the right end of the ø29 genome and transcribed early after infection from promoter C2, encodes a protein of 130 amino acids, p16.7. Wilfried J.J. Meijer and Alejandro Serna-Rico showed that p16.7 is an integral membrane protein and that the 22 N-terminal amino acids are required for its membrane localization. A derivative lacking the N-terminal transmembrane domain, p16.7A, forms dimers in solution and binds without sequence specificity to both single-stranded and double-stranded DNA. Meijer studied the in vivo role of p16.7 by constructing a *sus*16.7 mutant. ø29 DNA replication was delayed after restrictive infection with this mutant. Further insight into the in vivo role of p16.7 was obtained by comparing the localization of ø29 DNA replication in infected cells by immunofluorescence in the presence or absence of the protein. The redistribution of replicating ø29 DNA from the initial replication site to various sites surrounding the nucleoid was dependent on p16.7.

Protein p16.7A, which is purified as a dimer, forms multimers both in vitro and in vivo and interacts with the TP. In vitro multimerization is enhanced in the presence of DNA. Daniel Muñoz-Espín showed that the 70-amino-acid C-terminal domain of p16.7, p16.7C, constitutes the functional part of the protein because it forms dimers and multimers and binds to the DNA and to the TP, multimerization being a key factor for DNA binding. Recently, in a collaboration between my laboratory and the laboratories of Juan Luis Asensio and Armando Albert, the solution and crystal structure of the p16.7C dimer have been determined. Protein p16.7C defines a novel dimeric six-helix fold. Multimerization of p16.7C dimers is mediated by a large protein surface that is characterized by self-complementarity. More recently, the crystal structure of a tridimeric p16.7C-DNA complex was also determined by Armando Albert. This structure reveals the multimerization mode of the protein and provides insights into the organization of the phage genome at the membrane of the infected cell.

Other Viral Proteins Involved in ø29 DNA Replication: Proteins p17 and p56

Nonsuppressor *B. subtilis* cells infected with a *sus*17 ø29 mutant had a reduced ø29 DNA synthesis. Paola Crucitti showed that p17 overproduced by a gene 17–containing plasmid, obtained by Vladimir Benes, interacts

with protein p6 and stimulates the binding of the p6 to the ø29 DNA ends. More recently, Víctor González-Huíci has shown that protein p17 participates in the second step of ø29 DNA ejection into *B. subtilis* by pulling into the cell the 35% of the phage DNA that remains after the first (push) step.

Early gene 56, located at the left side of gene 1, encodes a protein of 56 amino acids, p56. By using in vitro chemical crosslinking and affinity chromatography, Gemma Serrano-Heras and Alicia Bravo found that uracil-DNA glycosylase (UDG), an enzyme involved in the base excision repair pathway, is a cellular target for protein p56. The addition of purified p56 to *B. subtilis* extracts inhibited the endogenous UDG activity. Moreover, extracts from ø29-infected cells were deficient in UDG activity. We suggested that inhibition of the cellular UDG by protein p56 is a defense mechanism developed by ø29 to prevent the action of the base excision repair pathway if uracil residues arise in their single-stranded replicative intermediates (40). Protein p56 is the first example of a UDG inhibitor encoded by a nonuracil-containing viral DNA.

Phage-Host Interactions in ø29 Development

It has long been known that phage ø29 does not develop efficiently in *B. subtilis* that sporulates, which was why we first used *B. amyloliquefaciens*, which does not sporulate, and then *B. subtilis* 110NA spoOA^{-}. Indeed, the work carried out by Wilfried Meijer and Virginia Castilla-Llorente has shown that ø29 adapts its infection strategy to the physiological conditions of the infected cell to optimize its survival and proliferation. Thus, the lytic cycle is suppressed when the infected cell has initiated the process of sporulation and the infecting phage genome is directed into the spore to remain dormant until germination of the spore. Two host factors are key players in this adaptive infection strategy. The chromosome segregation protein SpoOJ, which has binding sites in the ø29 genome, as first shown by Vanishree Murthy, seems to be involved in the spore entrapment of the infecting ø29 genome. SpoOA, the master regulator for the initiation of sporulation, also has several binding sites in ø29 DNA and suppresses ø29 development by repressing the early promoters A2c, A2b, and C2 and by preventing activation of the late promoter A3 (17). In addition, Meijer and Castilla-Llorente showed, together with Daniel Muñoz-Espín, a novel function for SpoOA: It inhibits the DNA replication of both the ø29 genome and the *B. subtilis* chromosome. Thus, binding of SpoOA near the ø29 DNA ends prevents formation of the protein p6-nucleoprotein complex, inhibiting the initiation of ø29 DNA replication. At the *B. subtilis ori*C, binding of SpoOA to specific sequences prevents open complex formation. Thus, by binding to the origins of replication, SpoOA prevents the initiation step of DNA replication of either genome.

OTHER COLLABORATIONS

Before finishing the scientific account of the work carried out in my lab over the past 40 years, I would like to mention other scientists with whom I worked in the lab. Francisco Alvarado worked on the proteins of the ø29 particle, Esteban Domingo was involved in studies on the *B. subtilis* RNA polymerase, Vaclav Paces worked on ø29-like phages, and Juan Alonso isolated protein p4 mutants that were later shown to interact with DNA. Antonio Gómez, from the group of Rubéns López, Ernesto García, and Pedro García, sequenced the ends of phage Cp-1 DNA. Carmen Ibáñez and Lucía Herranz, who worked with José L. Carrascosa, cloned the ø29 connector protein and studied its binding to the viral DNA, respectively. Javier Vinós collaborated with Julio Gutiérrez to study the signals in ø29 DNA required for the in vitro initiation of replication. Diego Guirao worked with Cristina Escarmís on the replication of recombinant ø29 DNA molecules in *B. subtilis* protoplasts. Tiina Pakula, from Dennis Bamford's lab,

characterized the SSB protein of phage PRD1. Audrey King and Arjen B. Brenkman, from Peter van der Vliet's lab, studied the properties of the adenovirus DNA polymerase and the role of YXGG/A motif, respectively. Juan J. López-Rubio, from Montserrat Elías-Arnanz's lab, spent time in my lab purifying the *Myxococcus xanthus* RNA polymerase. Several technicians worked over the years in the ø29 group: Victoria Gijón, our first technician; Nieves Fonturbel; Pilar Zaragoza; and Marisa Nogal. Margarita Corral was our first secretary, followed by Carmen Hermoso. My gratitude goes to all of them. In addition, I would like to thank Germán Rivas for collaborating on the analytical ultracentrifugation experiments with proteins p6 and p16.7, Mauricio G. Mateu for helping determine the dimerization constant of protein p16.7, and Jeff Errington for allowing Wilfried Meijer first and more recently Daniel Muñoz-Espín to work in his lab to learn fluorescence microscopy techniques. I also thank Dwight Anderson and Bernie Reilly for their collaboration in putting together the two collections of ø29 mutants.

I hope I have not forgotten anybody whom I should have mentioned. If I did, I express my apologies beforehand.

FINAL REMARKS

I have dedicated most of my life to research. This work has given me much gratification. All the work carried out in my lab has been basic research, trying to unravel the molecular basis of phage ø29 development. But the basic research has led to an important application: the use of phage ø29 DNA polymerase for DNA amplification, both for rolling circle and whole-genome amplification. This is a good example of how performing basic research can lead to the discovery of applications that were not expected or foreseen.

I cannot forget the 23 years I taught molecular genetics at Madrid Complutense University, from 1968 to 1992. Teaching allowed me to interact with excellent students, many of whom came to my lab for a PhD thesis. In 1992 I was appointed Director of the Center of Molecular Biology "Severo Ochoa" and thus had to stop teaching, which I had been doing voluntarily (because I belong to the Spanish National Research Council, I do not have teaching duties).

The year 2007 is the 40th anniversary of our work on phage ø29, which began in Spain in 1967. We will celebrate this occasion with a scientific Symposium in which most of my students and collaborators will participate. We expect to have a great time. I am happy to say that, after 40 years of working with bacteriophage ø29, we still make new and interesting findings, and a total of 46 PhD theses have been made on the phage ø29 system. Most of the students we have had over the years, both on the ø29 and on the African Swine Fever virus projects, have attained independent scientific positions in Spain, are doing excellent work and, in turn, are teaching new students. I am proud to say that molecular biology came of age in Spain quite a few years ago thanks, in part, to my and Eladio's initial teachings.

DISCLOSURE STATEMENT

I am not aware of any biases that might be perceived as affecting the objectivity of this review.

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