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MEMORIES OF A SENIOR SCIENTIST

A passion for research

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Abstract

During my postdoctoral training in Severo Ochoa's laboratory, I determined the direction of reading of the genetic message and I discovered two proteins in *Escherichia coli* involved in the initiation of protein synthesis. After my return to Spain, I have been working with the *Bacillus subtilis* phage ϕ29. We discovered a protein covalently linked to the 5′ DNA ends that is the primer for the initiation of ϕ29 DNA replication. We also found that the phage-encoded DNA polymerase has unique properties such as processivity and strand displacement activity. These properties, in addition to its high fidelity, have made the ϕ29 DNA polymerase the ideal enzyme for DNA amplification, both for rolling circle and whole-genome amplification. I am happy to say that the work carried out in my laboratory has been possible thanks to many brilliant students and collaborators, most of whom have become high quality independent scientists.

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I was born in 1938 in Canero (Spain), a very small village located in Asturias, on the north-west coast of Spain. One year later, my parents moved to Gijón, also located in the coast of Asturias, where I spent my childhood and early youth. Since I wanted to pursue a university career, something encouraged by my parents, when I finished the Baccalaureate, I had to spend one year doing the so-called pre-university studies and I had to decide whether I wanted to follow a scientific or a humanistic career. I decided to go into the scientific field and one year later I had to choose the specific university studies I wanted to follow. I liked both medicine and chemistry. I decided to go to Madrid, to the Complutense University, to take a course that was common for both careers. After I finished my first year, I decided to study chemistry. In the third year, I was fascinated by the laboratory work in organic chemistry. I enjoyed the many hours we spent at the laboratory and I thought that, in the future, I would like to do research in organic chemistry. But that summer, in Gijón, I had the luck to meet Severo Ochoa, and to attend a conference he gave on his work. Ochoa was a brilliant speaker, and I was fascinated by his talk. Since my father was a very good friend of Severo, besides being relatives-in-law (an uncle of Severo Ochoa was married to an aunt of my father), I had the chance to talk to him about my

future interests. I had not yet studied biochemistry and he promised to send me a biochemistry book. I was very excited when soon after I received the book *General Biochemistry* by Fruton and Simmonds, dedicated by Severo Ochoa. In addition, after that summer, I started to study biochemistry at the University. When I finished my University studies I had decided to do a PhD in biochemistry. Ochoa advised me to do my PhD thesis in Madrid with Alberto Sols, an excellent biochemist. Then, after my PhD thesis, I could go to his laboratory in New York for a postdoctoral training. Ochoa wrote me a reference letter for Alberto Sols who accepted me since he could not refuse a request made by Severo Ochoa, who at that time had already obtained the Nobel Prize. Thus, in January 1961, I started my PhD thesis in Alberto Sols' laboratory on carbohydrate metabolism, working on glucose phosphate isomerase from yeast.

At the end of our studies in chemistry, in 1960, I became the fiancé of a very brilliant student, Eladio Viñuela. Early in 1961, Eladio also started to work with Alberto Sols. His PhD thesis dealt with yeast phosphofructokinase, and he demonstrated its allosteric properties. In addition, he discovered a new enzyme, the liver glucokinase, that converts glucose into glucose-6-phosphate, and disappears in fasting and in diabetic rats. I joined Eladio in these studies and we found that the glucokinase activity reappears in the diabetic rats after insulin administration and in the fasted rats by refeeding. These results were published in the *Journal of Biological Chemistry* [1, 2].

Thanks to a fellowship that I obtained from the Juan March Foundation, we were able to marry in 1963, and in August of 1964, we went to Severo Ochoa's laboratory at the New York School of Medicine. We arrived in New York just in time to attend the International Congress of Biochemistry, where Leder and Nirenberg presented their latest results on the use of trinucleotides of specific sequence for the binding of the different amino acyl-tRNAs to the ribosomes. This resulted in the final unravelling of the genetic code that completed the work carried out in Ochoa's, Nirenberg's and Khorana's laboratories.

My initial research project in Ochoa's laboratory consisted of determining the direction of reading of the genetic message; that is, whether the reading was in the 5′–3′ or in the 3′–5′ direction. We used a cell-free protein synthesis system that consisted of a high-speed supernatant of *Lactobacillus arabinosus*, with low nuclease activity, and ribosomes from *Escherichia coli* that had been washed with 0.5 M NH_4Cl. As messenger RNA, we used synthetic polynucleotides that contained the AAC codon at the 3′ or 5′ end. When we used the polynucleotide $5'(A)_{24}AAC3'$, we obtained the incorporation of the amino acids lysine and asparagine, the latter being at the carboxyl end [3]. When the AAC triplet was located at the 5′ end, the asparagine was incorporated at the amino end [4]. Taking into account that the direction of protein systhesis takes place from the amino to the carboxyl end, the results obtained indicated that the direction of reading of the genetic message was from the 5′ to the 3′ end.

Afterwards, I started to work on the translation of the *E. coli* phage MS2-RNA using a high-speed supernatant from *E. coli* and the ribosomes washed with 0.5 M NH_4Cl. As expected, this system was active when I used polyA as messenger but, to my surprise, it was inactive with MS2-RNA. When I precipitated the 0.5 M NH_4Cl ribosomal wash with ammonium sulfate and added the fraction to the system, I recovered the activity with MS2-RNA. When I used the polynucleotide $5'AUG(A)_{24}3'$ prepared by Wendell Stanley Jr., I found that, as with MS2-RNA, this messenger was not active with the washed ribosomes but the activity was recovered when I added the ammonium sulfate fraction. Since the triplet AUG at the 5′ end of a messenger codes for formyl-methionine, this 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 showed that the two factors that I had purified from the ribosomal wash were required for such a binding. I called these factors F1 and F2 (later known as iF1 and iF2). Thus, this result clearly demonstrated that proteins F1 and F2 were required for the initiation of protein synthesis [5, 6].

After three exciting and fruitful years in Severo Ochoa's laboratory, Eladio and I decided to go back to Spain to try to develop in our country the molecular biology that we had learnt. An important decision was which kind of project we wanted to carry out in Spain, taking into account the difficulties we were going to find to do research in our country. In the summer of 1966, we had taken a Phage Course at Cold Spring Harbor and we thought that a phage could be a good model system to start our research. We choose the *Bacillus subtilis* phage ϕ29, shown in Dwight Anderson's laboratory to have a small size and a relatively complex structure [7]. Thus, it seemed to be a good model system to study the morphogenesis of the phage as well as the mechanisms of transfer of the genetic information as replication and transcription. Thanks to a grant we obtained from the Jane Coffin Childs Memorial Fund for Medical Research, we could start our work in Madrid.

We started our ϕ29 work in September 1967 at the Center of Biological Research of the Spanish National Research Council (CSIC), of which we had been appointed research scientists. As very little was known about phage ϕ 29 at that time we had to start from the very basic knowledge of the phage; to do the genetics isolating

conditional lethal mutants (temperature-sensitive and suppressor-sensitive), and to characterize the structural proteins of the phage as steps previous to the study of the morphogenesis of the phage particle, to isolate the phage DNA and to study its transcription.

Fortunately, soon after our arrival to Spain, the first predoctoral fellowships were awarded and we could start the work with several brilliant and enthusiastic students that made important progress in the knowledge of the molecular biology of the phage. In particular, I would like to mention the very important finding that ϕ29 has a protein covalently linked to the 5′ ends of the DNA, the so-called terminal protein (TP) [8, 9]. This finding led us to the study of the replication of ϕ29 DNA as the main topic in our laboratory, work that still continues.

We found that the TP is the primer for the initiation of ϕ29 TP-DNA replication catalyzed by the viral DNA polymerase, giving rise to the TP-dAMP initiation product that is further elongated by the same DNA polymerase to produce full-length ϕ29 DNA in vitro. The product of the viral gene 6 stimulates the in vitro initiation reaction under conditions in which the DNA ends are closed (high salt and/or low temperature). The ϕ29 DNA polymerase is unique since it is able to catalyze not only the polymerization step of replication, but also the initiation step. In addition, it has proofreading 3′–5′ exonuclease activity as well as two intrinsic properties such as very high processivity (>70 kb) and strand displacement capacity, which made it an ideal enzyme for DNA amplification [10]. Indeed, by using the ϕ29 proteins TP, DNA polymerase, protein p6 and the SSB protein p5, we were able to amplify in vitro small amounts (0.5 ng) of the 19,285-bp-long ϕ29 TP-DNA by three orders of magnitude (0.5 μg) after 1 h of incubation at 30°C. By transfection experiments, we showed that the infectivity of the in vitro amplified ϕ29 DNA was identical to that of the natural DNA obtained from virions [11].

Phage ϕ29 has given me much satisfaction. Through more than 40 years of work with this small phage, we obtained many important results from a basic point of view. But, in addition, the basic research with the replication system, in particular with the ϕ29 DNA polymerase, led to a relevant biotechnological application: the use of ϕ29 DNA polymerase for DNA amplification, both for rolling circle and whole-genome amplification [12, 13].

Also very rewarding were the 23 years (1968–1992) that I taught molecular genetics at Madrid Complutense University where I had very good students, some of which came to my laboratory to carry out their PhD theses.

I would like to remember that every 4 years, from 1980 till 1996, I organized in Salamanca (Spain) an EMBO Workshop on Bacteriophages. The best phage workers from Europe and the US attended the meetings.

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

In the early 1970s, Eladio decided to start a new project, the study of the molecular biology of African swine fever virus. The idea was twofold: on the one hand, he would work on a topic very important in Extremadura (Spain), his homeland. On the other, this would allow me to be an independent researcher in the ϕ29 work. Eladio established a powerful group that became a leader in the field. I was lucky and worked hard, I had many brilliant students, and Eladio helped me continuously. I am proud to say that molecular biology came of age in Spain quite a few years ago thanks in part to the initial teaching carried out by Eladio and myself.

Two years ago, in 2007, we celebrated the 40th anniversary of our work on phage ϕ29 with a scientific symposium in which most of the students and collaborators who had been involved in the ϕ29 work participated. I am happy to be able to say that after 42 years of work on ϕ29 we still make new and interesting findings, and a total of 50 PhD theses have been made on the ϕ29 system.

Before finishing, I would like to stress the fact that the work carried out in my laboratory is the result of the dedication and ideas of the many people who have worked in the ϕ29 group. I express my deepest gratitude to all of them, in particular to those who have helped me in the supervision of the work.

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Thanks are due to my parents for allowing me to develop a professional career, and to my brother and sister, also scientists, for their continuous support; to my friends for their advice and friendship; to our daughter Lucía, who has always supported me in my dedication to research; to my teachers: Alberto Sols and Severo Ochoa, from whom I learned biochemistry and molecular biology, respectively. And especially thanks to Eladio Viñuela, who was my husband, colleague, and always a teacher for me. Eladio, who is no longer with us, is most responsible for the success of my work.

I dedicate these memories to Eladio.

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