CELL FUNCTION AND DISEASE

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PREFACE

The new experimental tools and approaches of modern biology have allowed us to better understand many fundamental properties of the eukaryotic cells. These significant discoveries have drastically changed the diagnostic and therapeutic approaches of modern clinical practice. On April 18-22, 1988, an International Symposium on Cell Function and Disease was held in Monterrey, Nuevo León, México, aimed at reviewing some of the most recent advances made in the following five areas: Genes and Human Diseases; Cellular and Molecular Pathology; Infectious Diseases; Brain Transplants and the New Approaches and Techniques with Potential Application to Cell Function and Disease. This book is based on the contributed papers of the symposium. To underline the importance of the clinical approach to the study of cell function and disease a section on this subject was added at the end of the book. The chapters in this volume include contributions by some of the leading scientists of the international scientific community and México.

During the course of this international conference, numerous discussions were held by the local and international representatives of the scientific community concerning the creation of an International Center of Molecular Medicine aimed at stimulating further interaction between molecular biologists, biochemists, biophyscists and clinicians. Such ideas received the endorsement and support of the Director General of the United Nations Educational and Scientific Organization (UNESCO), Federico Mayor, the Governor of the State of Nuevo León, Jorge Treviño, and the Secretary of Health of México, Guillermo Soberon. In this context, then, this conference and these proceedings represent the first tangible results of the International Center of Molecular Medicine.

The organizers of this conference wish to acknowledge the President of México, Miguel de la Madrid, the Director General of UNESCO, Federico Mayor, the Governor of Nuevo León, Jorge Treviño and the Secretary of Health of México, Guillermo Soberon for inaugurating the conference.

We owe special thanks to the sponsors of the symposium: UNESCO, the Government of the State of Nuevo León, the National Council for Science and Technology (CONACYT), the University of Nuevo León (UANL), the Monterrey Technological Institute (ITESM), the International Biomedical Institute (Bari) and the National Foundation for Cancer Research.

In addition, we express appreciation to the other members of the organizing committee comprised of A. Azzi, H. Barrera, R. Drucker-Colín, G. Elizondo, C. Hazlewood, A. Kotyk, J. Kumate, S. Papa, B. Pullman, T. Slater and P. Ts'o.

We were also fortunate to have the help of many persons of the scientific and local community in the Monterrey area. In particular we express appreciation to Mrs. Angelina Decanini de Viesca for organizing the social program, to Mr. And Mrs. Eugenio and Eva Garza Laguera, Mr. And Mrs. David and Yolanda Garza Laguera and Mr. And Mrs. Generoso and María Elena Villareal, for hosting delegations of the visiting scientists. Also we thank colleagues and students at the Autonomous University of Nuevo León and the group of the Monterrey Technological Institute lead by Mr. Carlos Jiménez and integrated by Raúl Morales, Adrían Herrera, Alberto Sada, Leonel Dignowity, Bernardo Robles, Cristina Riojas, Esther Riojas, Jorge Salinas, José Bosco and Mauricio Belden, who took care of the visiting scientists and who helped with the numerous local arrangements. We appreciate the skillful administrative capacity of J. Gonzalez Miller and his staff as well as the group led by Professor Pamanes and José Díaz Chacón in supporting the logistical arrangement. Finally, we especially thank Ms. Martha T. Riojas for her help with all of the details that are part of running a conference and for her assistance with the editorial work of this book.

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INTRODUCTION

Nucleic acids and proteins are major protagonists in the continuously ongoing drama of life[1]. According to the now classical dogma, almost always respected and followed, DNA makes RNA, RNA makes protein, and protein closes the circle by providing activator and control elements necessary for proper DNA and RNA operation. In addition, proteins (in the form of enzymes) provide the more mundane function of catalyzing a variety of metabolic reactions, which culminate in the synthesis of vital components for the molecules of life.

Though proteins and nucleic acids interact closely together, they are basically different. Globular proteins, synthesized as linear polypeptide chains on the ribosome, quickly fold into stable three-dimensional structures, sometimes associate in dimers or higher units composed of identical or nonidentical subunits, occasionally form multienzyme complexes, capable of performing series of consecutive reactions with preordained space and time-coordinated activity. The globular, somewhat flexible protein subunit, stabilized by a variety of noncovalent and covalent - disulphide - bonds, extends a few nanometers into space; on it the catalytic active site can be identified as well as other accessory binding sites and surfaces capable of associating with other protein or nucleic acid structures. Throughout its lifetime in the cell the protein molecule maintains its structure and pursues its function, until it is replaced by an identical macromolecule following natural turnover, in keeping with a preassigned lifespan.

Nucleic acids carry the genetic information and control instructions for the complete organism, from the lowest unicellular to the highest eukaryote including man, encoded in a linear nucleotide code. A single DNA chain of a chromosome of a higher eukaryote, for instance man, composed of about 2×10^8 bp (base pairs), extends about 7 cm in length, though its width is not more than 2 nm. To maintain its integrity and to fit into the narrow confines of the nucleus in the cell, the DNA molecule must thus be highly folded and packaged into chromosomal structures. This packaging, in eukaryotic systems, is achieved with histone and sometimes non-histone proteins, in a broad hierarchy of structures. In the packaged form DNA is not active and the message it carries cannot be read. To allow the processes of transcription and replication, it is thus necessary to unfold selected portions of the chromosomal structure which can eventually be refolded, in a resting phase of the cell cycle of chromosomal activity. The necessity to achieve a quick transition from a totally folded to a partially unfolded chromosomal structure prescribes the necessity of potential lability associated with these structures. Folding

and unfolding of chromatin is believed to be due to postsynthetic modifications such as acetylation of non-structured histone tails, phosphorylation, ubiquitinization or the binding and release of non-histone high mobility group (HMG) proteins.[2] In chromatin, isolated as chromosomal fragments from nuclear structures by the use of nucleases or site specific restriction enzymes, the transition from more to less folded structures is mimicked *in vitro* by a decrease in the concentration of salt,[3] yet it is by no means established that the structure of chromatin at low concentrations of salt, far away from physiological conditions, is related to the physiologically unfolded form in a meaningful way.

In 1973 it was found that limited endonuclease action in rat liver chromatin led to distinct particles which were then defined as nucleosome core particles consisting of 145 bp complexed with eight core histone molecules (two each of the H2a, H2b, H3 and H4 core histones).[4,5] The structure of the nucleosome core particle has been characterized by X-ray diffraction[6] and controversies around its validity may be due to flexibility and structural rearrangements of the protein core in various solvent systems.[7] The chromatosome,[8] the complete chromatin repeating unit, consists of 160 bp of DNA, wound in two superhelical turns around the core histone octamer, locked at the common entry and exist point by an H1 (H5, or other linker histone variant) linker histone. The histones, in particular the H4 and H3 histones, are amongst the evolutionary most conserved proteins known[2]. The first stage in DNA compaction is the formation of the DNA core histone linker histone complex, representing a succession of nucleosomes separated by linker DNA stretches. The linker DNA stretches vary within a given organism around an average value which is different in different organisms.

A commonly accepted view, which has found its way securely into biochemistry textbooks is the concept of the folding of the linear chains of nucleosomes from a flexible random coil structure at low ionic strength (the 10 nm LOS lower order structure - 10-11 nm is the large diameter of the flat cylinder nucleosome), into the 30 nm diameter rigid HOS higher order solenoid.[9] In the Finch and Klug solenoid the pitch of the single-start helix is 11 nm (the height of the nucleosome stacked head-to-head), there are about six nucleosomes per turn of the helix, their faces roughly radially parallel to the helix axis (a conclusion strengthened by electric dichroism studies[10]). At best this structure is an idealization considering the variable and irregular linker length and conflicting opinion with respect to the dependence of the nominal 30 nm higher order structure diameter on linker length.[11,12] The controversies surrounding the basic chromatin structures were summarized in 1986[13,14,22] and further discussion here will deal with more recent developments. In a soccer game one side usually wins, but sometimes there is a draw. I would conclude that while we seem to be getting a better view of the lower order structure at low ionic strength, whose relevance to biological function is moot, our views on the higher order structure appear to be locked in a temporary draw.

THE STRUCTURE OF BULK CHROMATIN

Bulk, or total genomic, chromatin obtained by limited nuclease action on nuclei and subsequent lysis is prone to aggregation and precipitates with increase in concentration of monovalent and multivalent cations.[15-17] It is an extremely sensitive material and its properties often depend on ways in which it has been handled both before and after isolation. Chicken erythrocyte chromatin is more stable and less prone to attack by endogenous nucleases than chromatin from other sources and has therefore become a favorite system for study. At monovalent salt concentrations of about 0.15 M non-histone proteins are mostly removed and it is believed that the basic structure formed by the DNA and the core and linker histones is maintained. It is therefore essential to maintain the integrity of these components and to avoid migration of the linker histones which dissociate earlier than the core histones with increasing ionic strength. In general two philosophies have been developed to study the structure of the isolated bulk chromatin. In one approach, attempts were made to obtain a highly soluble subfraction of chromatin

and then to apply a range of physical methods in solution, at variable conditions of electrolyte type and concentration under conditions in which aggregation was completely avoided.[15] Once clear, reproducible and interpretable results are obtained, the question relating to relevance cannot be avoided. For electrolyte concentrations approaching physiological conditions the question is less severe than, as already mentioned, for the very low electrolyte environment, sometimes investigated. In the other approach, deriving from attempts to study chromatin *in situ* in intact cells,[18] the guiding concept was not to isolate chromatin fragments but to recreate conditions of cellular aggregation in the capillary test-tube, by choosing conditions and concentrations leading to benign orientation of the aggregated chromatin strands, without application of an external orientating force.[19] Previously attempts of producing oriented chromatin fibers had failed because of the already mentioned inherent instability of these structures.

Aided by modern image analysis to improve the quality of their X-ray diffraction patterns, Widom and Klug[19] succeeded in identifying equatorial and meridional reflections which essentially confirmed the validity of the Finch and Klug[9] solenoid. Earlier,[10] support for the solenoid structure was derived from electric dichroism studies, undertaken at millimolar $MgCl_2$ concentrations, in the absence of other electrolytes. Conflicting claims as to the sign and the size of dichroism corresponding to full orientation following application of electric or hydrodynamic fields have plagued these activities and are discussed in a recent work.[20]

Solution studies at increasing electrolyte concentrations comprising sedimentation in the ultracentrifuge,[21] quasielastic and total intensity light scattering[15] pointed to compaction with increasing ionic strength or addition of $MgCl_2$, though the inherent low resolution of these methods precluded meaningful analysis in terms of a significant unique molecular model. The considerably lower wavelength of X-ray and of neutrons leads to increased resolution in solution scattering studies[22] and significant experimental advantages (reliable extrapolation to lower scattering vectors) accrued from the use of synchrotron radiation for X-rays[23] and sophisticated neutron scattering facilities.[24] A major conclusion from these latter studies is that the low electrolyte LOS is not the disorganized 10 nm coil as previously believed but has enough stiffness and structure to yield a considerably larger cross-sectional radius in a cross section scattering plot, not much different from that of the HOS into which it folds quickly and easily. A true transition from a 10 nM LOS into a 30 nm HOS thus does not appear to be a good description of the folding process. A major alternate model to the Finch and Klug solenoid is the crossed-linker double start helix[11], which has lost the central hole characterizing the solenoid, and claims linker-length dependent diameter. The latter conclusion, as well as the correct value of the mass-per-unit length have not satisfactorily been settled from either scattering or STEM measurements.[24] In an experimental X-ray scattering study in our laboratory, in which the lowest scattering angles were not available, we could show that the chromatin folding process could be simulated by the compaction of a wormlike coil or a collection of rigid cylinders.[22] Koch *et al.* [12] confirmed that convolution of the wormlike coil with the nucleosome scattering yielded the features observed in the low-angle scattering curve.

Presently it does not appear that methods in hand will allow satisfactory resolution of the points under discussion. Much of the uncertainty may be due to the irregularity of the structure and it is therefore of significant interest that a construct has been achieved[25] with nucleosomes spaced regularly along a defined-size self-repeating DNA. So far it has not been possible to attach a properly located linker histone to this construct and recreate a complete well-behaved chromatin chain. This leads to the inevitable conclusion that the DNA entrance and exit sections on these reconstituted nucleosomes may not be properly arranged, as it has been shown that H1 can be removed from native chromatin and reaffixed with complete restoration of the folding properties in the HOS.[26] When linker histone is carefully removed from native chromatin the LOS behaves like a 10nm coil and loses the ability to fold into the HOS, which is regained upon readdition of H1.

THE β-GLOBIN GENE

The structural study of total genomic bulk chromatin represented a necessity arising from the fact that relatively large amounts of material are required for physical studies. Difficulties encountered in the determination of the crystal structure of the nucleosome core particle, heterogeneous with respect to DNA composition, were overcome by genetically engineering a uniform DNA fragment, which folds precisely into a crystallisable nucleosome.[27] A similar result has not yet been achieved for a chromatin fragment. The concentration of single-copy genes is minute (two copies of the gene per nucleus) yet current molecular biology technology has made it possible to study chromatin structure near an expressed gene, in distinction to the study of essentially inactive bulk chromatin.[28]

DNA fragments comprising the adult β-globin gene from chicken erythrocytes have been isolated, cloned, sequenced and used as probes in the study of β-globin chromatin. A basic property of chromatin near an expressed gene is its sensitivity and hypersensitivity to nuclease action[29] though nucleosomes from the β-globin gene were found to be similar in structure to nucleosomes from bulk chromatin.[28] Hypersensitivity most probably arises from the fact that DNA stretches surrounding the gene are not covered by nucleosomes but interact with transcription factors relating to gene expression.[30] In consequence of this structural feature it could be shown by careful analysis of migration in sucrose gradients of random nuclease digests of chicken chromatin that at 100 mM NaCl β-globin chromatin from erythrocytes remains partially unfolded whereas ovalbumin gene containing chromatin from the same tissue folds well.[31] At lower electrolyte concentrations (25 mM NaCl) both chromatin samples migrate identically. The opposite result was obtained when the two genes were isolated from oviduct chromatin. Kimura *et al.* [32] had earlier shown that an EcoR1 6.2 kb (kilo base pair) chicken erythrocyte fragment, enclosing the β-globin gene, sediments more slowly than bulk chromatin fragments of similar size, whereas ovalbumin and α2-collagen gene fragments in erythrocyte chromatin and β-globin in spleen chromatin sediment with bulk chromatin fragments of the same DNA size. More recently,[33] in an elaboration of these observations it was shown that the specific retardation of the chicken β-globin chromatin fragments cannot be reversed by adding extra linker histones to native chromatin. Globin and bulk chromatin behave identically upon unfolding by removal of linker histones or lowering of the ionic strength. The original difference in sedimentation can be restored by readdition of the linker histones and elevation of the ionic strength. Cleavage of the EcoR1 chicken globin fragment to remove the hypersensitive ends, in the 5' and 3' flanking regions, leads to a fragment sedimenting normally.

Both active and less transcriptionally active genes are organized in a nuclear matrix in loops of varying sizes[34] and it not clear to date whether transcribing RNA polymerase moves along these loops in the transcription process, or remains bound close to the base of the loops.[35,36] For partial unfolding to allow transcription, temporary removal of linker histone is necessary, though the question whether an RNA polymerase transcribes through a nucleosome core without its transient release from the DNA has received opposing answers.[37,38] There have only been limited attempts to purify unique genes as chromatin[39,40] and additional efforts in this direction will help to answer some of the questions raised.

Although DNA binding proteins have been isolated from prokaryotic organisms and we would expect its chromatin organization to be much simpler than that of eukaryotes, very little is known with certainty about the structure and modulation of bacterial chromatin.[41]

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MOLECULAR GENETICS OF WILMS' TUMOR

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Based on present rates, some type of cancer will affect three out of ten individuals in the United States in his or her lifetime (Amer. Cancer Soc., 1988). The impact of cancer on the health of the population has made an understanding of the etiology and biology of malignancy a primary research concern. The role of chromosome abnormalities in tumorigenesis was first proposed by Boveri in 1914. The subsequent observation of the clonal nature of most tumors supported the notion that genetic alterations were critical in carcinogenesis. Thus, the identification, isolation, and characterization of genes involved in carcinogenesis has become an important approach for understanding cancer etiology and biology. Advances in the past decade in molecular biology, cytogenetics, and somatic cell genetics have allowed the localization and cloning of many human disease loci. The isolation of genes involved in carcinogenesis is confounded by the heterogeneous nature of neoplasia and the absence of a single gene that is clearly involved. One approach to circumvent these complications is to study cancers in which only one or a few genes are hypothesized to have a major role in tumorigenesis. Wilms' tumor (WT) is one such cancer.

Wilms' tumor is an embryonal renal neoplasm which affects about 1 in 10,000 children (Matsunaga, 1981). One or both kidneys can be affected with 5-10% of Wilms' tumors being bilateral. In ~8% of cases, Wilms' tumor is associated with aniridia or genitourinary anomalies (Breslow and

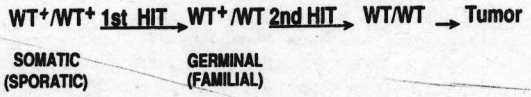

Fig. 1. Two-hit model for tumorigenesis as proposed by Knudson and Strong (1972). Two mutations are required for the development of a tumor. In somatic (sporadic) Wilms' tumor, both mutations are somatic. In germinal (familial) Wilms' tumor, an individual inherits the first mutation; only one subsequent somatic mutation is required for tumorigenesis.

Beckwith, 1982). Epidemiological studies have revealed that a vast majority of tumors occur sporadically with no family history of Wilms' tumor. About 1% of the cases, however, are familial, most often affecting siblings or cousins. Familial cases are more frequently bilateral than sporadic cases (Strong, 1984). In general, familial and bilateral cases are diagnosed at an earlier age (median of 25 months for bilateral tumors vs. 36 months for unilateral cases; Breslow and Beckwith, 1982). From these observations Knudson and Strong (1972) proposed that, like retinoblastoma, another pediatric tumor, the development of Wilms' tumor requires two mutations (Fig. 1). In nonheritable (somatic) WT, the initial mutation is a somatic event, whereas in heritable (germinal) WT the mutation is germinal. In both nonheritable and heritable WT, the second mutation is somatic. Because individuals have inherited the first mutation, only a single additional mutation may be sufficient for tumor development, leading to an earlier age of onset and the more frequent development of bilateral tumors. From the observed data on age of onset and laterality, it was estimated that in heritable WT, ~63% of carriers of the predisposing gene would be affected (Knudson and Strong, 1972).

GENETIC ALTERATIONS IN WILMS' TUMOR

Cytogenetic studies first indicated that the genomic location of at least one of the hypothesized mutations was at chromosomal band 11p13. Karyotypic analyses of patients with Wilms' tumor and aniridia (which is associated with Wilms' tumor in 1-2% of cases; Breslow and Beckwith,