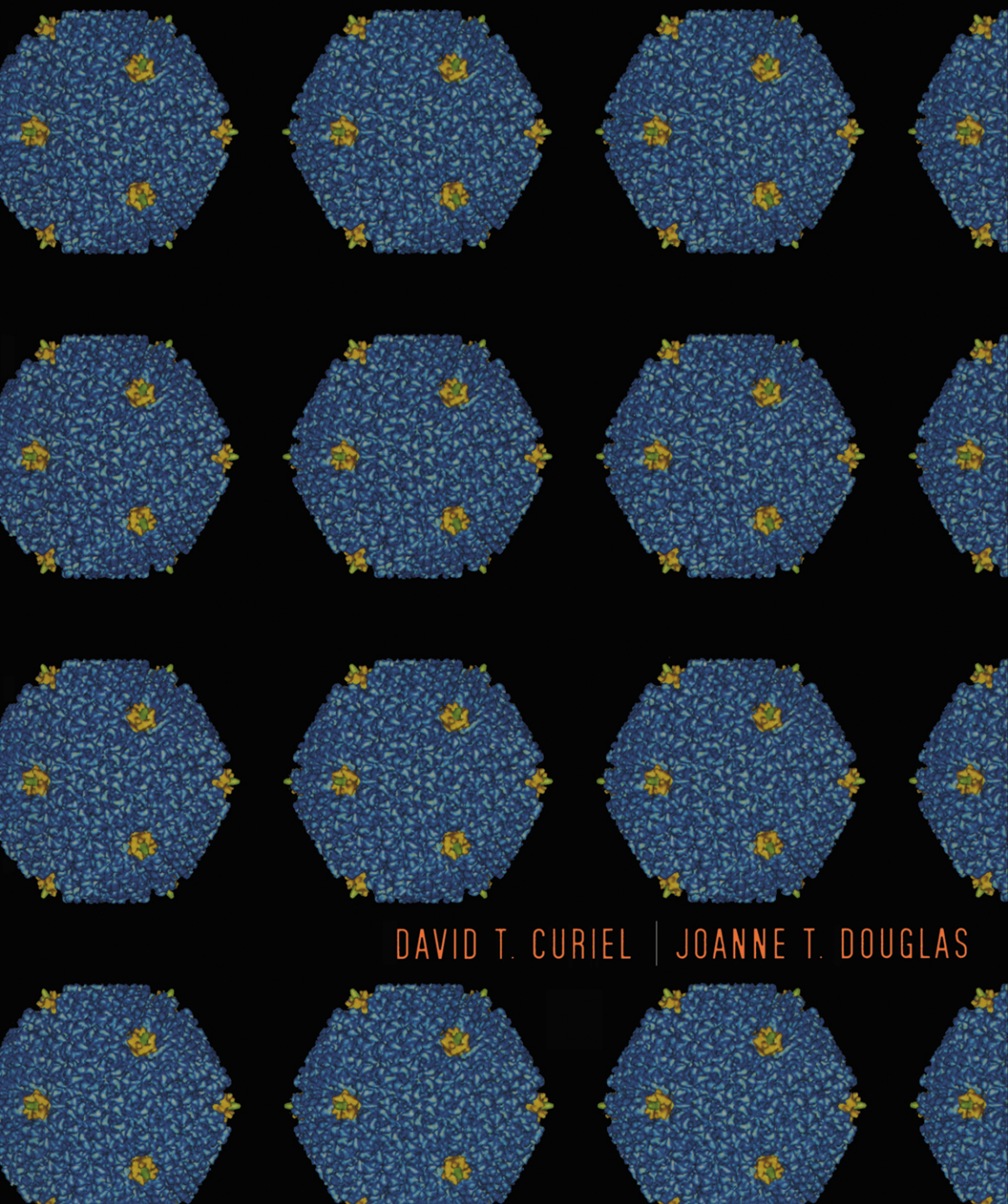
ADENOVIRAL VECTORS FOR GENE THERAPY



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Adenoviral Vectors for
Gene Therapy

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Adenoviral Vectors for Gene Therapy

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Preface

The number of human gene therapy clinical trials employing adenoviral vectors is expanding at an unprecedented rate. This increased use of adenoviral vectors has both fueled, and has in turn been fueled by, a parallel explosion in our knowledge of the biology of adenoviruses and their vectors. Moreover, there have been concomitant advances in associated technologies. It is therefore timely to review both basic and applied aspects of adenoviruses and adenoviral vectors in a single, comprehensive, multi-author volume.

The first few chapters focus on basic virology—the structure of adenoviruses and the biology of adenoviral infection and replication. Advances in our understanding of the parental virus have facilitated the rational design of adenoviral vectors for gene therapy. The construction, propagation, and purification of adenoviral vectors have benefited from a number of technological advances, as discussed in the next series of chapters.

In addition to the underlying biological features that favor their use for gene therapy, it is recognized that adenoviral vectors have suffered from a number of limitations. These limitations, together with strategies by which they might be overcome, are considered. Thus, separate contributions discuss approaches to target adenoviral vectors to specific cell types, as well as strategies to circumvent the host immune response. Replication-competent adenoviruses, which are increasingly being used as oncolytic agents for the treatment of cancer, are described. Other vectorological advances covered in this section include high capacity adenoviral vectors, xenogenic adenoviral vectors, and

hybrid adenoviral vectors, which combine the advantages of adenoviral vectors with beneficial features derived from other vector systems.

The next group of contributors describes the use of adenoviral vectors in animal models of human disease—cancer, genetic disease, and acquired diseases. These chapters discuss the lessons that have been learned from these model systems and their implications for the employment of adenoviral vectors in humans. Specific approval from the regulatory bodies must be obtained prior to the implementation of human trials, as detailed in the following chapter. Finally, the recognition of the need for noninvasive methods to monitor adenovirus-mediated gene transfer in human patients has predicated the development of novel imaging technologies.

In the aggregate, we have provided herein a comprehensive overview of adenoviral technology, both classical and novel. This update should provide an entrée into the field for the neophyte as well as a reference source for the practitioner.

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Adenoviral Vectors for Gene Therapy

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CHAPTER 1

Adenovirus Structure

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I. Introduction

The world got its first look at the icosahedral symmetry of adenovirus (Ad) in 1959 with published electron micrographs of negatively stained Ad5 [1]. In this classic work, Horne *et al.* were able to resolve the basic subunits and thus determine that the adenovirus capsid is composed of 252 subunits, 12 of which have five neighbors (pentons) and 240 of which have six neighbors (hexons). A few years later, Valentine and Pereira [2] published a striking electron micrograph of a single Ad5 particle, revealing the long protruding fibers that are characteristic of adenovirus. In analogy to what was known at the time about the role of phage tails, the authors correctly deduced that the adenovirus fiber might be involved in adsorption to the host cell surface. Since then electron microscopy has continued to play a role in our understanding of the structure of adenovirus and its interaction with αv integrins [3, 4]. In recent years X-ray crystallography has contributed atomic structures for the capsid proteins hexon [5, 6], fiber knob [7–9], and shaft [10], the fiber knob complexed with a receptor domain[11], and the virally encoded protease [12].

Our growing knowledge of adenovirus structure has already contributed to the field of vector design [13]. For example, initial attempts at modifying the C-terminal end of the fiber protein gave suboptimal results for gene delivery [14], while subsequent efforts utilizing knowledge of the fiber knob structure produced vectors with enhanced performance [15, 16]. Strategies for improving adenoviral vectors by making genetic modifications to capsid proteins and by designing hybrid vectors are discussed in later chapters. An understanding of adenovirus structure will be essential for these endeavors.

ADENOVIRAL VECTORS FOR GENE THERAPY

II. Molecular Composition

The approximately 50 known human adenovirus serotypes are classified into six subgroups, A–F, and all share a similar structure and genomic organization [17]. Adenovirus is an nonenveloped virus of ~150 MDa, composed of multiple copies of 11 different structural proteins, 7 of which form the icosahedral capsid (II, III, IIIa, IV, VI, VIII, IX) and 4 of which are packaged with the linear double-stranded DNA in the core of the particle (V, VII, mu, and terminal protein). For clarification of the nomenclature, note that most of the Ad polypeptides were named based on their position on a polyacrylamide gel. The highest molecular mass protein band turned out to be a complex of components, and consequently there is no polypeptide I in adenovirus. Also note that polypeptide IIIa was not originally resolved as a separate band; however, it is a distinct structural protein. In addition to the capsid and core components, approximately 10 copies of the adenovirus protease are incorporated into each virion [18].

For many icosahedral viruses, determination of a crystal structure has resolved outstanding molecular composition issues. In the case of adenovirus, there is as yet no atomic structure for the intact virion. In 1985, a preliminary X-ray crystallographic density map of the Ad2 hexon showed that the capsomer was a trimer of polypeptide II with a triangular top and a pseudohexagonal base [19]. Together with the early electron microscopy of the intact virion [1], the crystallographically observed hexon symmetry fixed the copy number of polypeptide II at 720 in the Ad virion. The stoichiometry of eight other structural proteins (III, IIIa, IV, V, VI, VII, VIII, and IX) was inferred by careful sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analyses of radiolabeled virions ([^{35}S] methionine) using hexon as the standard [20]. After adenovirus protease cleavage sites were found in the sequences of polypeptides IIIa, VI, and VIII [18], changing the number of methionines in the mature proteins, their predicted copy numbers were revised [21].

The molecular stoichiometry indicated that there is symmetry mismatch in the Ad penton [20]. Symmetry mismatches are not unheard of in icosahedral viruses. One example is SV40, which has pentamers of VP1 at sites of both local fivefold and sixfold symmetry in the crystal structure [22]. The conformationally flexible C-termini of VP1 are able to adapt to the position of the pentamer within the SV40 capsid. In the case of adenovirus, three copies of polypeptide IV form the fiber and five copies of polypeptide III form the penton base. The fiber and penton base together compose the penton, which sits at the fivefold symmetry axes of the icosahedral capsid. Microheterogeneity in the Ad penton base has been offered as an explanation for the symmetry mismatch [20].

More recently a reversed-phase high-performance liquid chromatographic (RP-HPLC) assay was developed in order to more fully characterize the Ad5 proteome [23]. N-terminal protein sequencing and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy were used to identify each component protein contributing at least 2% to the total protein mass of the virus. Peaks for the fiber protein, which contributes only 1.8% of the total protein mass, as well as the terminal protein and the protease, were not identified. The mass of the remaining structural proteins was determined to within ±0.1%. Their copy numbers were estimated using hexon as the standard and with the exception of the copy number for the core polypeptide VII, which was significantly reduced, the new copy numbers are in good agreement with the SDS-PAGE numbers [20, 21]. The precise mass measurements confirmed the proteolytic processing of polypeptides IIIa, VI, VII, VIII, and mu and interestingly cleaved precursor products of all but polypeptide IIIa were found to be present in the purified Ad5 virions.

III. Structure of the Intact Virion

In 1991 the first structure of an intact Ad particle was determined by cryo-electron microscopy (cryo-EM) and three-dimensional image reconstruction methods [24]. The technique of cryo-EM was developed in the mid-1980s by Dubochet and colleagues [25] for imaging viruses and other macromolecular assemblies in a native-like, frozen–hydrated state. Since then it has proven to be a powerful approach for studying icosahedral viruses and it has been applied to numerous members of over 20 different viral families [26]. The method involves placing a droplet of concentrated virus on an EM grid layered with a holey carbon film (carbon with holes 1–10 μm in diameter), blotting with a piece of filter paper to leave a thin (~1000 Å) layer of water and sample stretched across the holes of the grid, and then plunge freezing into a cryogen such as ethane slush chilled by liquid nitrogen. This rapid freezing causes formation of vitreous (amorphous) ice rather than crystalline ice. Formation of normal crystalline (hexagonal) ice would be harmful to the biological sample because of its expansion relative to liquid water. After cryo-freezing the sample grids are maintained at liquid nitrogen temperature to preserve the vitreous state. Transmission electron micrographs are collected using a low dose of electrons to avoid significant radiation damage to the frozen, unstained sample. The real power of the technique lies in the fact that many particle images can be computationally combined to generate a three-dimensional density map [26–28].

In the early 35-Å-resolution reconstruction of Ad2, the features of the icosahedral protein capsid were clear and its dimensions without the fiber were measured as 914, 884, and 870 Å along the five-, two-, and threefold symmetry

axes, respectively [24]. The reconstruction showed the trimeric shape of the hexon, the pentameric shape of the penton base, and a short portion (~88 Å) of the fiber shaft. The full-length fiber, ~300 Å long including the knob at the distal end, was occasionally visible in cryo-electron micrographs. Comparison of these particle images with projections of modeled full-length fibers indicated that the knobs were not positioned as would be expected if the fibers were straight. This suggested that the Ad fibers in the intact Ad2 particle are bent or flexible. Electron micrographs of negatively stained Ad2 fibers show a bend close to the N-terminal end, which binds the penton base [29]. A pseudo repeat of 15 residues was noted in the central section of the Ad2 fiber sequence [30] and later analysis of the fiber sequences from a variety of Ad serotypes revealed a range of 6–23 pseudorepeats in the shaft [31]. A long, nonconsensus repeat at motif 3 was proposed to induce a bend in the shaft of many Ad serotypes [31]. The idea that the fiber is bent for many Ad serotypes is consistent with both negative-stain electron micrographs [29] and the fact that only a short rigid portion of the Ad2 fiber shaft was reconstructed [24].

A more recent cryo-EM reconstruction of Ad2 [3] is shown in Fig. 1 (see color insert) with modeled full-length fibers. Reconstructions have now been published of Ad2 at 17-Å resolution [32], Ad5 [33], Ad12 [3], Ad2 complexed with a Fab fragment from a monoclonal antibody directed against the integrin-binding region of the penton base [34], both Ad2 and Ad12 complexed with a soluble form of αvβ5 integrin, the internalization receptor for many Ad serotypes [3], and a fiberless Ad5 vector [33]. The capsids of these Ad serotypes appear quite similar, with only subtle differences observed in the size and flexibility of the surface protrusions of the hexon and penton base [3].

IV. Structure of the Capsid Components

A. Hexon, Polypeptide II

Crystal structures have been published for hexon of serotype Ad2 [5] and Ad5 [6], two members of subgroup C. The sequences of these hexons (967 amino acids for Ad2, 951 for Ad5) are closely related with 86% amino acid identity. Both structures show that the monomer has two eight-stranded β-barrels at the base and long loops that intertwine in the trimer to form a triangularly shaped top (Fig. 2). The high degree of interlocking observed between the monomers might explain why an adenovirus-encoded 100-kDa protein is required for trimer assembly [35]. In the trimer the six β-barrels, two from each monomer, form a ring with pseudohexagonal symmetry that allows for close packing with six neighboring capsomers in the icosahedral capsid. Regions of the electron density for the Ad2 hexon, refined to 2.9-Å resolution,

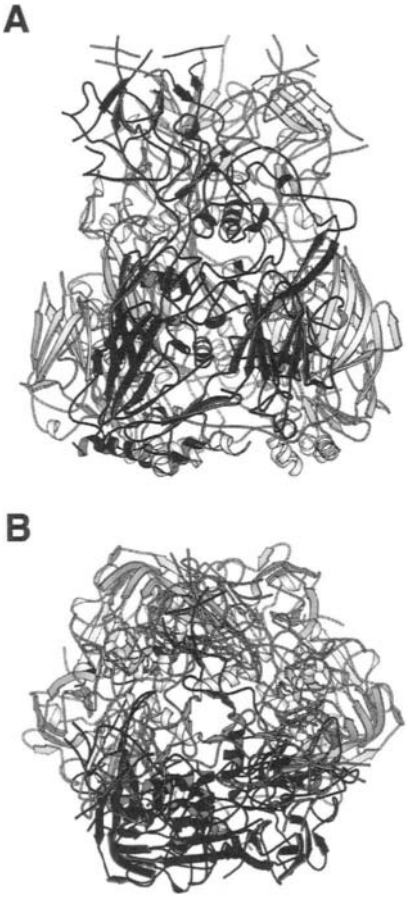

Figure 2 The crystallographic structure of the Ad5 hexon trimer [6] with one monomer shown in black (PDB ID: 1RUX [90]). (A) A side view showing the two β-barrels near the bottom of the black monomer. Note that there are several gaps in the atomic model at the top of the molecule. (B) A top view revealing the pseudohexagonal shape of the bottom of the trimer. This figure was generated with the program MOLSCRIPT [91].

were unclear and gaps were left in the atomic model. During refinement of the Ad5 hexon to 2.5-Å resolution, significant changes were made in the atomic model involving reassignment of greater than 25% of the sequence. In light of this result and the high homology between the two hexons, it has been suggested that the Ad2 atomic model should be revised [6]. The most significant change was a shift of the first 130 amino acids leaving a gap of just four residues at the N-terminus of the Ad5 structure vs an N-terminal gap of 43 residues in the initial Ad2 model.

Revision of the hexon structure has cleared up several mysteries in the literature. First, a comprehensive comparison of hexon sequences from serotypes in all six human subgroups as well as bovine and mouse serotypes found seven hypervariable regions [36]. Alignment with the Ad2 hexon structure indicated that five regions were in exposed loops as expected, while two regions were buried. The Ad5 hexon structure now shows all seven hypervariable loops exposed on the top of the molecule [6]. Second, trypsin cleavage sites were identified at Arg-142 and Arg-165 in Ad2 [37] and these are now located in the exposed top of the hexon molecule [6]. Similarly a pH-dependent cleavage site for the proteolytic enzyme dispase was found somewhere between residues 135 and 150 of the Ad2 hexon [38]. In the original Ad2 hexon structure this stretch was buried and far from the top of the molecule. In the Ad5 hexon structure this region is likely exposed on the molecule, although it is in an unmodeled region of the structure [6].

The Ad5 structure places a previously buried highly acidic stretch of residues, 133–161 for Ad2, at the top of the molecule and accessible to solvent [6]. The acidic region is also found in the Ad5 hexon sequence, but not in those of Ad9, Ad12, or Ad37. In the Ad8 hexon sequence there is a longer, slightly basic insertion at this position [36]. It has been suggested that the acidic stretch may create an electrostatic repulsion between the exterior of the Ad2 or Ad5 virion and acidic cell surface proteins [39]. Others have proposed that perhaps the acidic region plays a role in tissue tropism for the subgroup C viruses [40].

B. Penton Base, Polypeptide III

In the absence of a crystal structure for the penton base, structural information on this protein comes mainly from cryo-EM reconstructions of the dodecahedron formed by Ad3 pentons [41] and intact Ad virions of various serotypes [3, 32–34]. Alignment of the known penton base sequences from subgroups A, B, C, and E shows high homology throughout the protein except for a central variable length region that contains the nearly always conserved Arg-Gly-Asp (RGD) sequence, residues 340–342 for Ad2 [4,42]. The Ad2 and Ad5 penton bases (571 residues each) have among the longest variable RGD regions [4,43,44]. The RGD sequence, utilized for interaction with cellular αv integrins [4,45], is lacking from the enteric Ad40 and Ad41 serotypes of subgroup F [46]. Presumably these two serotypes don't interact with αv integrins during viral cell entry.

Site-directed mutagenesis of the Ad2 penton base has indicated particular residues that are important for various functions including pentamerization and stable fiber-penton base interaction [47]. While recombinantly expressed Ad2 penton base is known to self-assemble into homo-pentamers, two mutations in the N-terminal portion of penton base, R254E and W119 H, and several