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RECOMBINANT DNA TECHNOLOGY IN ADENOVIRUS RESEARCH

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Summary. — A brief description of methods for cloning adenovirus genomic DNA and cDNA is presented, the use of recombinant clones in adenovirus research is illustrated by several examples, with particular emphasis on recent informations concerning the structure and function of adenovirus transforming genes.

The discovery of molecular cloning methods (isolation of specific DNA fragments from complex genomes and their multiplication in bacterial and other technical innovations (blot hybridization, DNA sequencing etc.) have induced a great progress in molecular biology — including molecular virology. In this paper we would like to give an outline on the alternative methods for cloning of adenoviruses, and to bring different examples for the use of the recombinant clones, with emphasis on investigations of the transforming region of human adenoviruses.

Cloning of genomic adenovirus DNA fragments

The adenovirus genome consists of a linear, double stranded DNA molecule of approximately 35 000 base pairs. The molecular cloning of the *internal* adenovirus DNA fagments is a routine procedure. Both the adenovirus and the vector DNA (lambda phage or plasmid) is digested with the same restriction endonuclease, ligated, and *E. coli* is transformed with the hybrid molecules. The recombinant bacterial clones are characterized by hybridizing the DNA from colonies or plaques with the appropriate ^{32}P-labelled adenovirus DNA fragments and/or by digesting the isolated recombinant plasmids or phages with restriction endonucleases to determine the size and orientation of the insert (Engler *et al.*, 1981; Palkonyay *et al.*, 1982; McKinnon *et al.*, 1982).

The *terminal* adenovirus DNA fragments cannot be cloned by the above mentioned simple procedure, because the linear, double stranded adenovirus genome possesses a 55K terminal protein attached to its 5'ends, which makes the 5'termini inaccessible to the ligation reaction. This blockage of the 5'ends cannot be abolished by extensive protease treatment, because a serine residue of the 5'terminal protein remains covalently bound to the 5'terminal dC residue (Challberg and Kelly, 1982). Brief exonuclease III and subsequent Sl-nuclease treatments however, remove the terminal 50—100 nucleotides generating unblocked flush ends on both sides of the DNA molecule (Byrd

et al., 1982; McKinnon *et al.*, 1982; Stow, 1981). As a next step, synthetic oligonucleotide "linkers" can be joinded to the fragments, so that the ligation of the fragments would be easier and the newly constructed recombinant plasmids could be redigested with the restriction endonuclease used for cloning. Alternatively, the blunt ended fragment can be ligated to a similarly blunt ended vector, but in this case the original inserted DNA fragment cannot be reisolated again by restriction endonuclease digestion.

The cloning of fragments of adenovirus DNA makes it possible to prepare a large quantity of pure DNA fragments without isolation and purification of a large amount of adenoviruses from cell cultures. The recombinant plasmid DNA can be used for detailed physical mapping, for DNA sequencing, for nick translation — to be used as a hybridization probe, or it can be bound or immobilized to nitrocellulose or other (chemically activated papers) filters to select mRNAs for in vitro translation or cDNA preparation. (For excellent description of these methods see Maniatis *et al.*, 1982). The cloned fragments can be used to introduce mutations (deletions or insertions) into the viral genome at specific sites. Partially digesting a recombinant plasmid with a restriction endonuclease having several recognition sites within the inserted fragment, religating it and transforming *E. coli* with this reaction mixture, clones containing plasmids missing certain fragments can be selected as deletion mutants. Alternatively, the cloned fragment cut by a restriction endonuclease having unique cleavage site within this DNA region can be subjected to limited Sl nuclease digestion followed by recircularization. Using this procedure a limited number of nucleotides can be removed from the DNA, started at the cleavage site of the restriction endonuclease used in the experiment. The mutant plasmids can be distinguished from the wild type, since they lost their unique cleavage site for the given restriction endonuclease (Fowlkes and Shenk, 1980).

Kapoor and Chinnadurai (1981) have developed a method to construct adenovirus mutants cotransfecting permissive cells with a recombinant plasmid, which contains a mutagenized viral fragment and two viral segments derived from both ends of the viral genome, each of which have overlapping sequence homologies with the cloned viral segment. After cotransfection, a double recombination event took place in the cell within the overlapping regions resulting the new, mutant adenovirus genome. An alternative approach for generating mutations in a given cloned fragment of adenovirus applies transposable drug-resistance elements, transposons (Mc Kinnon *et al.*, 1982). The advantage of this method is that transposons can be inserted into a wide variety of sites in the plasmid DNA, it makes possible to obtain a series of mutants having insertions in several different sites of the adenovirus genome. Since transposons carry a functioning antibiotic resistance gene (for example Tn5 carries a kanamycin resistance gene), the selection of colonies having the given transposon inserted is very easy using an agar plate containing the corresponding antibiotic. The insertions generated by the transposons can be mapped by restriction endonuclease cleavage of the plasmids.

Cloning of cDNA

The discovery that the eukaryotic cells and their viruses contain mRNAs consisting of sequences derived from non-contiguous segments of the DNA genome — that is the spliced structure of mRNAs — was originally made in the Ad2 system (Berget *et al.*, 1977; Chow *et al.*, 1977). Since that time a continuous effort has been made to identify the exact borders of the mRNA coding sequences (exons) and the sequences present at the splice points (exon-intron borders). Although the molecular mechanisms involved in splicing have not been understood yet, a large amount of information has been accumulated concerning the structure of mRNAs in the case of adenoviruses (for review see: Nevins and Chen Kiang, 1981) and in other systems as well. The possibility to make a DNA copy (cDNA) of the matured mRNA and clone it as a double stranded DNA fragment providing a large amount of pure material for sequencing enormously stimulated this analytical work. In the protocol most widely used for cloning cDNA the first step is the preparation of the intact, specific polyA + mRNA. The selection of the desirable mRNA is performed by hybridization of the RNA with specific denatured DNA fragments bound to a solid phase (nitrocellulose filter). The first strand of the cDNA copy is synthesized by reverse transcriptase, the mRNA is subsequently hydrolyzed with NaOH, then the second cDNA strand is made by DNA polymerase I using the first cDNA strand as a template primer. The single stranded cDNA loop is then removed by S1-nuclease digestion, and the double stranded cDNA copy of the mRNA is cloned in plasmid or phage vector by dC-dG or dA-dT tailing, by the addition of synthetic oligonucleotide linkers, or by blunt end ligation. Another possibility for cloning mRNA sequences is the direct insertion of the mRNA molecule and its single stranded DNA copy as a mRNA (cDNA hybrid into a plasmid molecule) (Zain *et al.*, 1979). The advantage of this procedure over the double stranded cDNA cloning is that the terminal sequences — which are often lost when the first DNA transcript is used as a templat-primer for the synthesis of the second strand — remain intact. The low efficiency of transformation by mRNA/cDNA hybrid (10^2 transformants/μg) is a drawback of the method. Alternative approaches for cloning cDNA are discussed in the review article of Gaubatz and Paddock (1982).

Structure and function of the transforming genes of human adenoviruses

The human adenoviruses can be divided into five subgenera (A to E) based on their DNA homology, G + C content, polypeptide structure and immunological properties. Enteric adenoviruses apparently fall into one or more further subgenera (Wigand *et al.*, 1982). The different serotypes (species) belonging to subgenus A are highly oncogenic, subgenus B serotypes are weakly and subgenus C and D serotypes are non-oncogenic in vivo; however, all human serotypes tested are able to transform primary rodent cells in vitro (Flint, 1980). According to transfection experiments with isolated specific DNA fragments, the left hand 12% of the adenovirus genome contains all the genetic information necessary for transformation of cells in culture

(Graham *et al.*, 1974; Van der Eb *et al.*, 1977; Shiroki *et al.*, 1977). This conclusion is further supported by the observations that all cell lines transformed by adenoviruses contain viral sequences homologous to the left-most 12% of the genome, these viral sequences are expressed in the transformed cells, and mutants mapped in this regionare unable to transform cells. This left terminal segment of the adenovirus DNA contains one of the four regions transcribed early during infection called El, which was subdivided into regions Ela and Elb based on the two transcription units localized here and transcribed in a rightward direction (Chow *et al.*, 1977) and on te genetic complementation groups defined by host range mutants (Jones and Shenk, 1979). DNA fragments containing only intact Ela region and mutants defective in Elb region (for example d1313 of Ad5) are able to transform cells incompletely (Shiroki *et al.*, 1979; Houweling *et al.*, 1980; Shiroki *et al.*, 1981). The partially transformed cells can be distinguished from the completely transformed ones on the basis of their fibroblast like elongated morphology, more slow growth, lack of ability to reach high saturation density, atypical T antigen distribution and lower T antigen concentration. However, both the partially and completely transformed cells have unlimited life span and they consist of aneuploid cells (Van der Eb *et al.*, 1980). During the last few years the transforming genes of the different human adenovirus serotypes, their biological properties and detailed molecular structure have been extensively studied and compared with each other using the whole methodical arsenal of the molecular biology — including recombinant DNA technology.

Structure and function of the Ela region

Three different spliced mRNAs are transcribed from the Ela region. The 12S and 13S mRNAs are transcribed early after infection. The 12S mRNA directs the synthesis of 54K and 42K proteins, while 58K and 48K proteins are translated from the 13S mRNA in vitro. According to the DNA sequence, these proteins are exceptionally rich in proline and glutamic acid (Perricaudet et al., 1980a). The third RNA encoded by the Ela region, the 9S RNA is produced predominantly late after infection and specifies the production of a 28K protein. The Ela regions of other serotypes of human adenoviruses are organized in a very similar way, although the size of Ela coded proteins was found slightly different in the case of Ad h 7 or Ad h 12 (Dijkema *et al.*, 1980; Perricaudet *et al.*, 1980c).

Studies with different deletion (dl) and host range (hr) mutants of adenoviruses have shown that a functional Ela region is required for the accumulation of early viral mRNAs (Nevins, 1981). In the absence of intact Ela region the other early genes (E1b, E2, E3, E4) are transcriptionally inactive. The transcription of E2 and E3 regions seems to be the most sensitive to the defect in the E1a gene, followed by E4, while E1b is the least sensitive (Osborne *et al.*, 1982). The major late promoter (at 16.4 m.u.) is also active early after infection, resulting in the transcription of L1-specific sequences (Shaw and Ziff, 1980) and the E1a function is required for its transcription early after infection, too.

In the presence of agents blocking protein synthesis (cycloheximide, emetin, anisomycin) both in cells infected with wild type Ad h 5, and cells infected with an Ad h 5 E1a mutant (dl 312), wild type levels of early viral transcripts have been measured (with the exception of E3 region mRNA); thus the inhibition of protein synthesis could mimic the E1a function. However, in the hr 1 Ad h 5 mutant, which has a point mutation between 2.8 and 3.5 m.u. preventing the synthesis of the 13S RNA specified 58K and 48K proteins, the early viral transcripts do not accumulate. The best explanation for this phenomenon has been offered by Nevins (1981) that a cellular factor with short half life prevented the activity of all early viral genes except of E1a and that an E1a-coded protein inactivated this factor. The inhibition of the cellular protein synthesis exerts the same effect on early viral genes as the E1a function, removing the cellular factor and abolishing the inhibition of early viral transcription. Such a cellular factor may not only suppress the early viral genes, but certain cellular genes as well. Therefore, its inactivation by the E1a-coded product would allow the expression of some cellular genes, which are otherwise inactive. This mechanism could play a role in the transformation caused by the E1 region. In fact, a 70K protein is induced in large amounts in wild type Ad h 5 infected, but neither in dl312-infected nor in uninfected cells (although with highly sensitive methods a very low amount of 70K protein had been detected in uninfected HeLa cells). Furthermore, in HeLa cells subjected to heat shock (exposure to 43 °C) a new 70K protein appears to be induced besides other changes of the polypeptide pattern. This 70K "heat shock" protein — as partial protease mapping data have revealed, is identical with the 70K protein synthesized in adenovirus infected cells, although the mechanism of its induction is not quite clear so far. The Ad h 5-transformed human cells (293 cells) which express constitutively the E1a and E1b viral genes, contain considerable amounts of this 70K protein.

The large increase in the production of the 70K protein is in each case the consequence of the induction of its transcription (Nevins, 1982).

Another function related to the E1a gene is the induction of host cell DNA replication and TK activity, the appearance of chromosome abberations in growing cells. This function is independent from the activation of other early viral promoters caused by the E1a gene, since some mutants, sub315 and sub316, which fail to induce cell cycle alterations, are able to activate the other viral early transcription units (Braithwaite *et al.*, 1983).

Structure and function of the E1b region

The E1b transcription unit of adenoviruses is located between 4.6—11.2 m.u. Two predominant mRNAs are produced from this region early in infection: the 13S RNA and the 22S RNA which has identical 5′ and 3′ ends as the 13S mRNA, but its intervening sequence is much shorter (Perricaudet *et al.*, 1980*a*). The third RNA (9S), which is synthesized only in small amounts, is specifying the structural protein IX and it is produced in higher amounts late in infection.

In Ad h 2 and Ad h 5, three major E1b-coded proteins have been detected besides several minor protein species. In the case of Ad h 2 53K, 19K and 20K proteins have been identified by Green *et al.* (1982); according to Esche *et al.* (1980) the estimated molecular weights of the same Ad h 2 proteins are slightly different : 57K, 15K, 18K. The 57K (53K) and the 20K (18K) proteins are translated probably in the same reading frame, since they share ^{35}S-methionine labeled peptides, but the smallest of the three predominant proteins, the 19K (or 15K) is not related to the 57K. Using the sera of animals bearing Ad2 or Ad5 induced tumours or using antisera against in vitro Ad2 or Ad5 transformed cells, the 57K and 19K proteins are precipitated, therefore they can be considered as adenovirus tumour antigens (Perricaudat *et al.*, 1980*b*). The 19K protein has been found in all adenovirus transformed cells tested, it can be obtained from the membrane fraction of both Ad2-trans- formed and lytically infected cells (Persson *et al.*, 1982). Certain Ad h 12 mutants (cyt mutants) — which are transformation defective and have considerably reduced tumorigenicity in hamsters — cause the degradation of both viral and cellular DNA upon infection. The cyt mutation maps in the E1b region, in cells infected with cyt mutants no 19K polypeptide can be detected, therefore the probable function of 19K protein is to prevent the activity of DNases (LaiFatt and Mak, 1982).

Cells transformed with adenovirus DNA fragments bigger than the leftmost 8% of the genome are tumourigenic when injected into athymic nude mice. However, when cells transformed with smaller DNA segments have been tested for in vivo tumourigenicity, the results were consistently negative. The failure of the transformed cells to give rise to tumours in animals correlated with the lack of the 19K E1b protein, thus probably the 19K tumour antigen is essential for the in vivo oncogenic ability of adenoviruses (Van der Eb *et al.*, 1980).

The presence of the 57K tumour antigen, which is a phosphoprotein, is also a general phenomenon, although in some transformed cells it has not been detected (Matsuo *et al.*, 1982). In transformed cells the 57K protein can be found in a complex with a 54K cellular protein. Interestingly, high levels of the same 54K cellular protein can be detected in cells transformed by a variety of agents (chemicals, irradiation, viruses, spontaneous transformation), in SV40-transformed cells physically associated with the SV40 large T antigen (Sarnow *et al.*, 1982), and in Epstein Barr virus transformed cells the EBNA has been found in close association with a 54K protein (Luka *et al.*, 1980). In cells productively infected with adenoviruses the 57K tumor antigen is present in a free form, the complexes have been observed only in transformed cell lines (Sarnow *et al.*, 1982).

Viral sequences in adenovirus-transformed cells and in tumour cells

In cells transformed by adenoviruses in vitro or derived from tumours induced by adenoviruses in vivo the viral DNA molecule or a part of it is integrated into the host cell genome. Ad h 12-transformed cells contain multiple copies of most or all of the viral genome, which are in most cases

colinear with the virion DNA (Doerfler *et al.*, 1980; Schirm and Doerfler, 1981). However, in cells transformed by Ad h 2 or Ad h 5 usually only a part of the viral genome is represented. Most frequently sequences corresponding to the central fragments of the viral DNA are deleted, the left terminal "transforming region" is always present, and a rather high proportion of Ad h 2- and Ad h 5-transformed cell lines contain sequences derived from both termini of the viral genome linked together directly (Vardimon *et al.*, 1981; Visser *et al.*, 1982) or in inverted orientation (Sambrook *et al.*, 1980; Vardimon *et al.*, 1981).

The relatively high frquency of the linkage of DNAs derived from the two termini of the adenovirus genome suggests the formation of circular intermediates in the process of integration. In fact, covalently closed circular adenovirus DNA molecules have been found in BRK cells infected with Ad h 5 hr1 mutant by Ruben *et al.* (1983).

In a rat liver epithelial cell line transformed by Ad h 12, although the adenovirus specific tumour antigens have been identified, no Ad h 12 specific DNA sequences have been detected (Paraskeva *et al.*, 1982). Kuhlman *et al.* (1982) studied an Ad h 12-transformed cell line. H1111, which had apparently lost all Ad h 12 sequences and the lack of virus specific DNA in this cell line was accompanied with a change from epithelial to fibroblast-like cell morphology, but the cells retained their ability to induce tumours in hamsters. Thus the transformation of cells by adenoviruses may occur as a "hit and run" action, that is, the viral sequences induce a profound change in the cells resulting the transformed state, but once it has happened, some characteristics of the transformed state will be maintained regardless of the presence of the viral information.

Conclusions

The development of methods of molecular biology, the introduction of recombinant DNA technology has induced a revolution in the adenovirus research. Our knowledge of the transforming region of adenoviruses has increased greatly over the last few years as a result of a great deal of work of several laboratories. However, the main mechanisms leading to transformation and tumourigenesis still have to be elucidated.

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