JOURNAL OF VIROLOGY, Sept. 1997, p. 6576–6581 Vol. 71, No. 9
0022-538X/97/$04.00+0

Molecular Characterization of the Type-Specific γ-Determinant Located on the Adenovirus Fiber

BRITTA EIZ AND PATRICIA PRING-ÅKERBLOM*

Institut für Virologie und Seuchenhygiene, Medizinische Hochschule Hannover, 30623 Hannover, Germany

Received 10 February 1997/Accepted 13 June 1997

The fiber knob carries the type-specific γ-antigen which can be demonstrated in hemagglutination inhibition tests. In order to characterize the γ-determinant we selected subgenus DI adenovirus serotypes 9 and 19 (Ad9 and Ad19) which exhibited 29 amino acid exchanges in the knob domain. Like all subgenus DI adenoviruses they showed a complete hemagglutination pattern with rat and human erythrocytes. We constructed a total of 14 chimeric Ad9/Ad19 and Ad19/Ad9 fiber proteins, which possessed fiber knobs with progressively exchanged Ad9 and Ad19 amino acids. Furthermore, we created 39 fiber proteins with distinct amino acid exchanges in the knob regions by primer-directed mutagenesis. The proteins were expressed in *Escherichia coli* and tested in hemagglutination and hemagglutination inhibition tests. From our results we can conclude that the type-specific γ-determinant is not restricted to a distinct region on the adenovirus fiber knob but is composed of at least 17 amino acids. Most of the amino acids contributing to the Ad9 and Ad19 γ-determinants are located on the fiber knob loops.

Human adenoviruses are nonenveloped, double-stranded DNA viruses with an icosahedral capsid (9). The fiber, located at each of the 12 vertices of the capsid, plays a crucial role in attaching the virus to specific receptors on the cell surface (2, 16), thereby being of significant importance for viral infectivity and tissue tropism. Furthermore, the fiber is the viral hemagglutinin.

The fiber protein can be divided into three domains as proposed in the original model of Green et al. (4). The conserved N terminus contains the sequences responsible for association with the penton base as well as the nuclear localization signal (8, 15). A rod-like shaft of variable length contains repeats of a 15-amino-acid β-structure, with the number of repeats ranging from 6 in serotype 3 adenovirus (Ad3) (22) to 22 in Ad2 (4). A conserved amino acid sequence (TLWT) marks the boundary between the repeating units of the β-structure in the shaft and the globular knob domain (24). On the basis of the architecture of the viral capsid and of the fiber polypeptide, it is likely that the knob domain of the fiber contains the receptor attachment site. It has recently been demonstrated that the Ad2 and Ad5 fiber knob domains interact with HeLa cell viral receptors (5, 11). Furthermore, it has also been shown that the Ad5 knob can block virus infection (5) and that the receptor-binding specificities of adenovirus fibers can be altered by exchanging the knob domains (2, 23).

The fiber also carries the type-specific γ-antigen (14, 25). It determines, together with the ε-antigen of the hexon, the serotype specificity of an adenovirus. The existence of the type-specific determinant was first suggested by the hemagglutination inhibition (HI) test and by using monoclonal antibodies against the native fiber proteins of Ad2. To further elucidate the localization of the γ-determinant, we previously constructed truncated and chimeric fiber genes of Ad9, Ad15, and Ad19, all belonging to subgenus D and showing no HI cross-reactivity. In subsequent HI tests we could clearly show that the type-specific γ-determinant is located on the fiber knob (3).

Since relatively little is known about the molecular structure of the γ-antigen, we were interested in developing a more detailed understanding of the amino acids involved in the antigen-antibody binding. We therefore constructed a total of 14 chimeric Ad9/Ad19 and Ad19/Ad9 fiber proteins which possessed fiber knobs with progressively exchanged Ad9 and Ad19 amino acids. Furthermore, we created 39 fiber proteins with distinct amino acid exchanges in the knob regions. The proteins were expressed in *Escherichia coli* and tested in hemagglutination (HA) and HI tests.

Xia et al. (28, 29) recently published the crystal structure of the Ad5 fiber knob domain. A sequence comparison of the knob domains of several different adenovirus serotypes suggests an overall similarity in the structure (28, 29). While conserved residues occur in the β-sandwich motif, most of the surface loops are variable. A sequence alignment of the Ad5, Ad9, and Ad19 fiber knob domains allowed the localization of the amino acids involved in HI.

MATERIALS AND METHODS

Construction of recombinant plasmids. The Ad9 and Ad19 fiber DNA sequences (17, 19) were used to evaluate primer pairs for amplification by PCR. Primers were designed to amplify the entire coding sequences of the full-length fiber genes. The forward primers (9B- and 19B-) lack the ATG initiation codon since the PCR products were subsequently cloned into the pQE expression vector, which provides an in-frame start codon. The reverse primers (9E and 19E) ended with the termination codon TGA. For cloning purposes, the forward primers contained a *Kpn*I restriction site and the reverse primers contained a *Bam*HI restriction site. The fiber genes of Ad9 and Ad19 are referred to as F9 and F19, respectively. PCR amplification was performed as previously described (17). Previously cloned Ad9 and Ad19 fiber genes served as DNA templates for PCR (17, 19). Amplified products of the expected size were cloned into pQE-32 (Qiagen) which had been digested with *Kpn*I and *Bam*HI for expression in *E. coli* cells. The nucleotide sequence of the cloned insert was determined by sequencing (21), and in each case a clone having a perfect match with the published sequence was selected. The expressed proteins possessed N-terminal affinity tags consisting of six consecutive histidine residues (six-His tags).

Construction of chimeric fiber genes. For progressively exchanging F9 fiber knob gene nucleotide sequences for F19 sequences, the F9 and F19 DNA was amplified by PCR with different primer pairs (F9 forward primer, 9B-; reverse primers, 9Ch1R to 9Ch7R; F19 forward primers, 19Ch1 to 19Ch7; reverse primer, 19E). The reverse primers used for the amplification of the F9 tail-shaft-

* Corresponding author. Mailing address: Institut für Virologie und Seuchenhygiene, Medizinische Hochschule Hannover, D-30623 Hannover, Germany. Phone: 49-511-5324310. Fax: 49-511-5325732. E-mail: Adeno@T-Online.de.

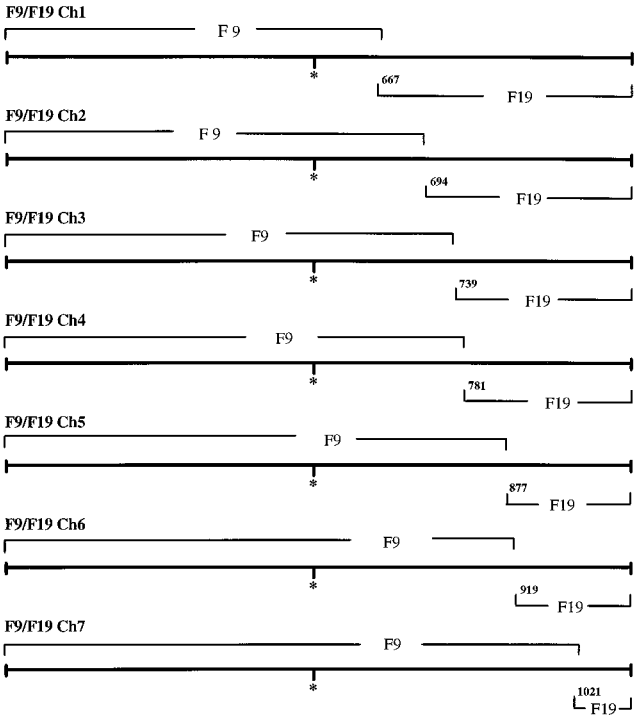

FIG. 1. Chimeric fiber constructs F9/F19 Ch1 to Ch7. The nucleotide sequence coordinates of the switch sites are indicated. *, beginning of the fiber knob domains.

partial knob domains were as follows: 9Ch1R, 5′-TACGACAATTAATGACA CAT-3′; 9Ch2R, 5′-GTTATTGATAATTTTGTACT-3′; 9Ch3R, 5′-AAAC AATAATTTAATGGTAA-3′; 9Ch4R, 5′-TTTACCAAGATTTGAAGATT-3′; 9Ch5R, 5′-AGGTTTTGGATAGGCTACCA-3′; 9Ch6R, 5′-TCCATAAACT ATATCTCTTG-3′; 9Ch7R, 5′-CCAACTAAAATCAAATGTGA-3′. The forward primers for amplifying the corresponding F19 knob regions were as follows: 19Ch1, 5′-GCAGAAAGGTACCACATCAT-3′; 19Ch2, 5′-AGACAAATCC AGAAATAAA-3′; 19Ch3, 5′-AATAAGAACGGAGTGCTTTT-3′; 19Ch4, 5′-GCTTATTGGAACTTTAGAAG-3′; 19Ch5, 5′-AGTAATTCTAAAAAATAT GC-3′; 19Ch6, 5′-ACTATATATCTTGGTGGAAA-3′; 19Ch7, 5′-TCCAAAAC CTATGAAAATGT-3′. The fiber gene chimeras were created by ligating the 9B-9Ch1R to 9Ch7R PCR products with the corresponding 19Ch1 to 19Ch7/19E PCR products (e.g., 9B-/9Ch1R with 19Ch1/19E). After ligation, a second PCR was carried out with the 9B-/19E primer pair. The PCR product was cloned into pQE-32. Following this protocol, seven chimeric F9/F19 fiber genes (F9/F19 Ch1 to Ch7; F for fiber, Ch for chimeric) were constructed in which parts of the F9 knob were progressively replaced by the corresponding parts of the F19 knob (Fig. 1). Besides the F9/F19 Ch1 to Ch7 fiber chimeras, we also created seven corresponding F19/F9 Ch1 to Ch7 fiber chimeras (primers not shown).

Construction of mutant fiber genes by primer-directed mutagenesis. Distinct amino acid exchanges in the F9 fiber knob domain were made by primer-directed mutagenesis (7). Two PCR products (i.e., 9B-/9MDis1R and 9MDis1/9E; M for mutated, Dis for biochemically distinct groups) overlapping in sequence and both containing the same primer-introduced mutation were generated and purified by gel electrophoresis. After denaturation and annealing of the two PCR products, a subsequent reamplification with primer pair 9B-/9E, resulting in the enrichment of the full-length mutated fiber gene (i.e., F9 MDis1), was performed. Figure 2 gives schematically the construction of mutated fiber gene F9 MDis1. A comparison of the F9 and F19 fiber proteins revealed 29 mismatches in the knob region (19 exchanges between biochemically distinct amino acid groups, changing the charges and/or the polarities of the corresponding sites; 10 exchanges between residues from biochemically identical amino acid groups) (Fig. 3). We therefore evaluated a total of 18 mutagenic primer pairs for the Ad9 fiber knob. For exchanging amino acids between biochemically identical amino acid groups 6 mutagenic primers pairs (9MId1 to 9MId6 and 9MId1R to MId6R; M for mutated, Id for biochemically identical groups) were synthesized, and for exchanging amino acids between biochemically distinct amino acid groups 12 mutagenic primer pairs (9MDis1 to 9MDis12 and 9MDis1R to 9MDis12R) were synthesized. The exact positions of the amino acid exchanges between biochemically distinct amino acid groups are marked in Fig. 4 (MDis1 to MDis12). Fibers with only one to three amino acid mutations between biochemically identical groups were named F9 MId1.1 to MId6.1, while fibers with only one to three amino acid mutations between biochemically distinct groups were named F9 MDis1.1 to MDis12.1; e.g., the F9 MDis2.1 fiber has only the mutation MDis2 with three amino acid exchanges (Fig. 4 and Table 2). Proteins showing progressively exchanged amino acids between biochemically identical or distinct amino acid groups were named F9 MId1 to MId6 or F9 MDis1 to MDis12, respectively; e.g., the F9 MDis2 fiber has the mutations MDis1 and MDis2 with a total of five amino acid exchanges (Fig. 4 and Table 2). In addition to F9 MDis2.1, we created three further proteins with three amino acid exchanges between biochemically distinct groups (F9 MDis7.2, with mutations MDis6 and MDis7; F9 MDis10.2, with mutations MDis9 and MDis10; and F9 MDis12.2, with mutations MDis9 and MDis12; Fig. 4).

The sequences of the primers used to create the primer-directed mutations were as follows (the reverse complementary primers 9MId1R to 9Id6R and 9MDis1R to 9MDis12R are not shown): 9MId1, 5′-CGTAGATGGTAGGT ACAAAATTATCAATAACAATAC-3′; 9MId2, 5′-CAATAACAATACTAAT CCAGCTATAAAAGGATTTACC-3′; 9MId3, 5′-GGAGTACTTTTAGACA ACTCAAATCTTGGTAAATC-3′; 9MId4, 5′-GGAACTTTAGAAGTGAAA ATTCAATTGTTTCAACAGCTTATG-3′; 9MId5, 5′-GCCTATCCAAAACC TAGTGCTGGCTC-3′; 9MId6, 5′-GTTTATGGAACTATCTACCTTGGTGG-3′; 9MDis1, 5′-CACATCTCCAAATTGCACAATTGCTCAGGATAAGGACT C-3′; 9MDis2, 5′-GTCATTAATTGTCGTAGCAGAAAAGTACCACATTAT C-3′; 9MDis3, 5′-CAAAATTATCAATAACAAGACTCAACCAGCTCTC-3′; 9MDis4, ′-AACAAGACTCAACCAGAACTCAAAGGATTTACC-3′;9MDis5, 5′-CTCAACCAGAACTCAAAAGTTTTACCATTAAATTATTG-3′;9MDis6, 5′-CATTAAATTATTGTTTAATAAGAATGGAGTACTTATG-3′; 9MDis7, 5′-CAAATCTTGGTAAAGCTTATTGGAACTTTAG-3′; 9MDis8, 5′-GGAA CTTTAGAAATGGAAATTCAATTATGTC-3′; 9MDis9, 5′-GAAATGGAAA TTCAAATATGTCAACAGCTTATG-3′;9MDis10, 5′-CTATCCAAAACCTA CCAATTCTAAAAAATATGCAAG-3′; 9MDis11, 5′-CCAGATCAACCAGC AGTCATTAAAACTACC-3′; 9MDis12, 5′-CATTTGATTTTAGTTGGTCC AAGACTTATGAAAATG-3′. Altogether, we created a total of 39 fiber knob mutants by applying primer-directed mutagenesis. The amplified full-length mu-

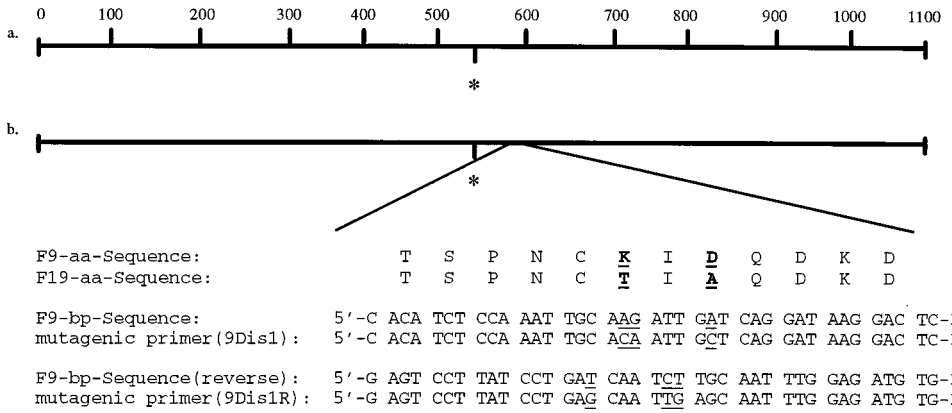

FIG. 2. The mutated fiber gene F9 MDis1. (a) Full-length adenovirus fiber gene; (b) recombinant fiber gene F9 MDis1. *, beginning of the fiber knob domain; bp, base pair; aa, amino acid. Mismatched amino acids are in boldface and underlined; mismatched nucleotides are underlined.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
F9	K	D	D	G	K	K	N	Q	A	L	G	D	E	M	E
	$+^2$	÷	÷	÷	*	$+^2$	$+^2$	*	÷	*	$+^1$	#	#	*	*
F19	T	A	A	E	R	H	K	N	E	I	S	N	K	L	D

	16	17	18	19	20	21	22	23	24	25	26	27	28	29
F9	S	S	N	E	I	M	T	A	G	N	V	T	A	V
	*	+	*	÷	+	*	*	+	-	*	≈	+	+	÷
F19	N	A	S	G	N	V	S	N	-	T	A	V	S	E

FIG. 3. Amino acid exchanges in the F9 and F19 fiber knobs. The numbers (1 to 29) designate the amino acid exchanges. Asterisks indicate exchanges between residues from biochemically identical amino acid groups (e.g., exchange of two nonpolar residues). Dashes indicate deletions. For exchanges between biochemically distinct amino acid groups (boldface), the symbols are as follows: +, exchange between polar neutral and nonpolar residues; $+^1$, exchange between polar neutral and polar acid hydrophilic residues; $+^2$, exchange between polar neutral and polar basic hydrophilic residues; ≈, exchange between nonpolar neutral and nonpolar hydrophilic residues; ÷, exchange between nonpolar and polar acid hydrophilic residues; #, exchange between polar basic hydrophilic and polar acid hydrophilic residues.

tated fiber genes of the expected size were obtained and cloned into pQE-32 which had been digested with *Kpn*I and *Bam*HI. The nucleotide sequence of the cloned insert was determined by sequencing (21), and in each case a clone having the correct mutation was selected.

Expression of the fiber constructs in *E. coli* cells. Expression of the recombinant fiber constructs was performed by standard techniques in *E. coli* cells (strain M15) after IPTG (isopropyl-β-D-thiogalactopyranoside) induction (final concentration, 2 mM) for 5 h. Cells from a 500-ml culture were harvested by centrifugation at 4,000 × *g* for 20 min, and the pellets were resuspended in sonication buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl). After being frozen and thawed, the cells were sonicated thoroughly. The fiber-containing supernatant was concentrated with microconcentrators (Centricon-30; Amicon). All recombinant fiber proteins were soluble.

The expression of each fiber protein was verified by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis according to standard techniques (10). The cell lysates were resuspended in sample buffer and then boiled to denature the trimers prior to SDS-PAGE (12% polyacrylamide gels). The gels were blotted onto a nitrocellulose membrane (Gibco BRL). The ^{RGS}His antibody (Qiagen) directed against the six-His tag served as the primary antibody. As a secondary antibody, an anti-mouse antibody, conjugated to alkaline phosphatase (Boehringer, Mannheim, Germany) was used. The color reaction was developed with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium; Boehringer).

HA and HI tests. The localization of the adenovirus γ-determinant was determined by HI testing using type-specific polyclonal rabbit immune sera against purified virions of Ad9 and Ad19. The antisera were provided by the National Reference Center for Adenoviruses. The HA and HI tests were performed essentially as previously reported (20) in a 1% suspension of rat and human erythrocytes, respectively, except that virions were replaced by recombinant fiber proteins. Ad9 and Ad19 virions served as positive controls.

For the HA tests, the virions or recombinant proteins were diluted in serial twofold steps in 96-well plates containing 25 μl of McIlvaine-NaCl buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 [pH 7.2]; diluted 1:50 with 0.87% NaCl). To each dilution, 25 μl of a 1% suspension of rat or human erythrocytes was added. The sedimentation pattern was determined after incubation for 1 h at room temperature. The last dilution showing a clear-cut complete agglutination pattern was considered to contain one HA unit. For the HI tests, serial twofold dilutions of the antisera in 25 μl of McIlvaine-NaCl buffer were mixed with an equal volume of two HA units of virions or recombinant proteins and incubated for 20 min at room temperature. Human erythrocytes (25 μl of a 1% suspension) were then added to each well, and the mixture was incubated for 1 h at room temperature.

RESULTS

Cloning and expression of the recombinant fiber proteins. PCR amplification of the Ad9 and Ad19 fiber genes yielded fragments of approximately 1,100 bp each. For the PCR products of F9 (9B-/9Ch1R to 9Ch7R and 9Ch1 to 9Ch7/9E) and F19 (19B-/19Ch1R to 19Ch7R and 19Ch1 to 19Ch7/19E) the expected sizes ranged from 100 to 450 bp for the Ch1 to Ch7 fragments and from 650 to 1,000 bp for the Ch1R to Ch7R fragments, respectively. After ligation of the fragments and reamplification of each of the recombinant fiber genes, F9/F19 Ch1 to Ch7 and F19/F9 Ch1 to Ch7 showed lengths of approximately 1,100 bp, a length which corresponds to those of full-length fiber genes F9 and F19. The 39 mutant fiber genes generated by primer-directed mutagenesis (F9 MId1 to MId6, F9 MId1.1 to MId6.1, F9 MDis1 to MDis12, F9 MDis1.1 to MDis12.1, F9 MDis7.2, F9 MDis10.2, and F9 MDis12.2) also showed the expected lengths. The fiber genes were directionally cloned into bacterial expression vector pQE-32 containing the ATG start codon. After IPTG induction the recombinant proteins were expressed in *E. coli* in the late stage of bacterial growth and were readily extracted as soluble proteins. The proteins were visualized as approximately 43-kDa polypeptides by denaturing SDS-PAGE (Fig. 5) and Western blotting (Fig. 6), the results of which agreed with the predicted molecular mass of 42.9 kDa based on the protein sequence (including the six-His tag). The band represents the monomer species of the fiber. No such polypeptide was observed before IPTG induction or in extracts from untransformed *E. coli* M15 cells grown under the same conditions.

HA and HI tests. Rosen divided the human adenoviruses into convent groups based on the ability of the viruses to agglutinate erythrocytes of various animal species (20). Further subdivisions based on the types of erythrocytes that different serotypes within a subgroup agglutinate have been suggested (13, 14). Adenoviruses of subgenus D can be subdivided

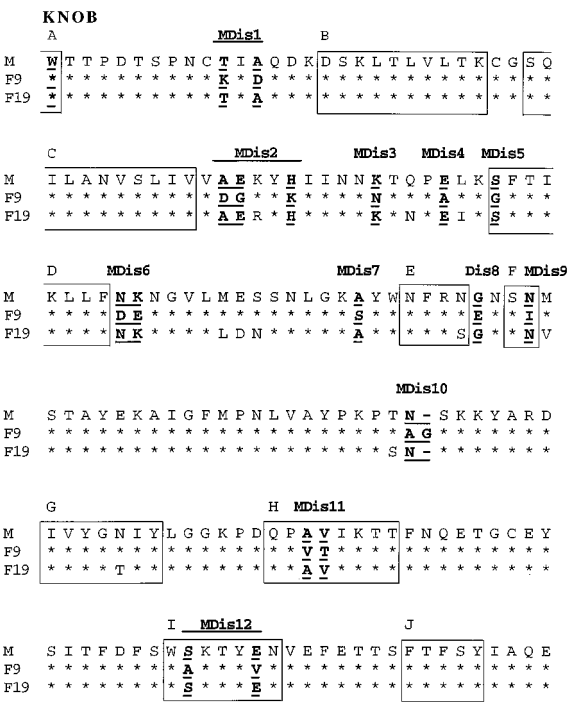

FIG. 4. Distinct amino acid exchange mutants—exchange of amino acids between biochemically different groups. The sequences were aligned to obtain maximum homology. Amino acids that are identical to those encoded by F9 are marked by asterisks; deletions are represented by dashes; M, mutated F9 sequence; MDis1 to MDis12, mutations. The boxes indicate the β-strands (A to J) in the types 9 and 19 adenovirus knob domains corresponding to the published structure of the type 5 adenovirus knob domain (28, 29). The regions (loops) between the β-strands were labeled by Xia et al. (28, 29) after the β-strands they connect, e.g. AB loop, CD loop (loops are not shown).

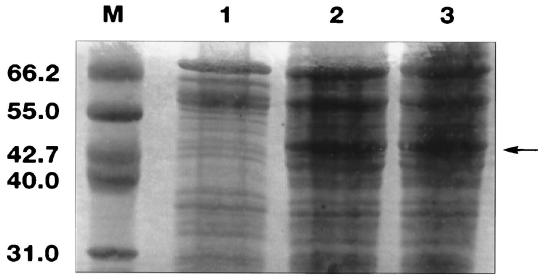

FIG. 5. Coomassie-stained SDS-PAGE gel of expressed recombinant fiber proteins. Lanes: M, molecular mass marker (the sizes of the markers are indicated on the left in kilodaltons); 1, *E. coli* M15 cell extract (not transformed); 2, fiber protein F9; 3, fiber protein F9/F19 Ch1. The arrow indicates the positions of the recombinant fiber proteins.

into three different clusters: cluster DI adenoviruses agglutinate rat and human erythrocytes, cluster DII adenoviruses only agglutinate rat erythrocytes, and cluster DIII adenoviruses frequently show no HA (6, 26). In HA tests, the full-length recombinant fiber proteins F9 and F19 showed the same HA pattern as that shown by the virions and native fibers of these serotypes. They agglutinated rat and human erythrocytes. The same results were obtained for all chimeric (F9/F19 Ch1 to Ch7 and F19/F9 Ch1 to Ch7) and mutated (F9 MId1 to MId6, F9 MId1.1 to MId6.1, F9 MDis1 to MDis12, F9 MDis1.1 to MDis12.1, F9 MDis7.2, F9 MDis10.2, and F9 MDis12.2) proteins. Besides being essential for subsequent HI tests, the HA tests indicated if sufficient amounts of functional recombinant fiber proteins had been produced by expression.

The adenovirus fiber knob carries the type-specific γ-determinant. The localization of the γ-antigen was determined by HI tests using type-specific sera against Ad9 and Ad19 (Tables 1 and 2). As expected from the HI reactions of the Ad9 and Ad19 virions (data not shown), the recombinant full-length fiber proteins of F9 and F19 showed no HI cross-reactivity (Table 1). As a first step towards identifying the γ-determinant, we constructed 14 chimeric fiber proteins. By progressively exchanging the amino acids of the F9 fiber knob for F19 sequences and exchanging the F19 fiber knob sequences for F9 sequences, we created the F9/F19 Ch1 to Ch7 and F19/F9 Ch1 to Ch7 chimeras, respectively. The fiber knob of F9/F19 Ch1, consisting mainly of F19 knob sequences, reacted only with Ad19 antisera, while the corresponding fiber knob of F19/F9 Ch1, consisting mainly of F9 knob sequences, reacted only with Ad9 antisera (Table 1). The same results were obtained for the corresponding F9/F19 and F19/F9 Ch7 proteins. All the other chimeric proteins with more than two amino acid exchanges (F9/F19 Ch2 to Ch6 and F19/F9 Ch2 to Ch6) showed HI cross-reactivity with antisera against Ad9 and Ad19 (Table 2).

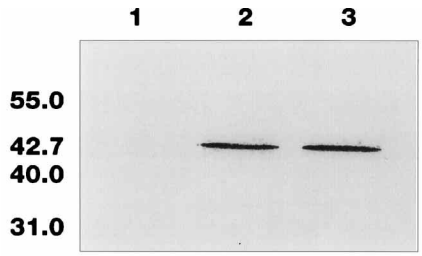

FIG. 6. Western blot. Lane 1, *E. coli* M15 cell extract (not transformed); lane 2, fiber protein F9; lane 3, fiber protein F9/F19 Ch1. The sizes of the markers are indicated on the left in kilodaltons.

TABLE 1. Fiber proteins without cross-reaction in HI tests[a]

Fibr protein	No. of exchanges[b] with amino acids of:		HI reaction with[c]:	
	F9 knob	F19 knob	Ad9 antiserum	Ad19 antiserum
F19	0	19	Neg.	Pos.
F9	19	0	Pos.	Neg.
F9/F19 Ch1	17	2	Neg.	Pos.
F9/F19 Ch7	2	17	Pos.	Neg.
F19/F9 Ch1	2	17	Pos.	Neg.
F19/F9 Ch7	17	2	Neg.	Pos.
F9 MDis1.1	2	17	Pos.	Neg.
F9 MDis3.1	1	18	Pos.	Neg.
F9 MDis4.1	1	18	Pos.	Neg.
F9 MDis5.1	1	18	Pos.	Neg.
F9 MDis6.1	2	17	Pos.	Neg.
F9 MDis7.1	1	18	Pos.	Neg.
F9 MDis8.1	**1**	18	Pos.	Neg.
F9 MDis9.1	1	18	Pos.	Neg.
F9 MDis10.1	2	17	Pos.	Neg.
F9 MDis11.1	2	17	Pos.	Neg.
F9 MDis12.1	2	17	Pos.	Neg.
F9 MDis1	2	17	Pos.	Neg.
F9 MDis11	17	2	Neg.	Pos.
F9 MDis12	19	0	Neg.	Pos.
F9 MId1.1	1	9	Pos.	Neg.
F9 MId2.1	2	8	Pos.	Neg.
F9 MId3.1	3	7	Pos.	Neg.
F9 MId4.1	2	8	Pos.	Neg.
F9 MId5.1	1	9	Pos.	Neg.
F9 MId6.1	1	9	Pos.	Neg.
F9 MId1	1	9	Pos.	Neg.
F9 MId2	3	7	Pos.	Neg.
F9 MId3	6	4	Pos.	Neg.
F9 MId4	8	2	Pos.	Neg.
F9 MId5	9	1	Pos.	Neg.
F9 MId6	10	0	Pos.	Neg.

[a] The reactions were performed with human erythrocytes.
[b] Amino acid exchanges between biochemically distinct groups.
[c] Pos., HI was detected; neg., no HI reaction. For all positive HI reactions, the titer was 1:128.

To further characterize the γ-determinant, we applied primer-directed mutagenesis and created F9 fiber proteins with distinct amino acid exchanges in the knob domains. By exchanging amino acids between biochemically identical groups a total of 12 mutated proteins (F9 MId1.1 to MId6.1 and F9 MId1 to MId6) were constructed. In HI tests, all these proteins (even proteins with more than two amino acid exchanges) only reacted with Ad9 antiserum; no inhibition reaction with Ad19 antiserum was observed (Table 1).

Mutated proteins F9 MDis1.1 and F9 MDis3.1 to MDis12.1, with only one or two exchanged amino acids, respectively, between biochemically distinct groups, showed no cross-reactions in HI tests (Table 1). As expected from the previous results, no cross-reaction was observed for proteins F9 MDis1, F9 MDis11, and F9 MDis12 (Table 1). F9 MDis1 (two mismatched amino acids from biochemically distinct groups) showed an HI reaction only with Ad9 antiserum, while F9 MDis11 and F9 MDis12 (17 and 19, respectively, amino acid exchanges between biochemically distinct groups) reacted only with Ad19 serum in HI tests. In contrast, proteins F9 MDis2 to MDis10 all showed cross-reaction in HI tests with Ad9 and Ad19 antisera (Table 2). Since we had already observed that three exchanged amino acids from biochemically distinct groups (F9 MDis2.1) lead to HI cross-reactivity (Table 2), we wanted to investigate if any three amino acid exchanges be-

TABLE 2. Fiber proteins with cross-reaction in HI tests[a]

Fiber protein	No. of exchanges[b] with amino acids of:		HI titer[c] for:	
	F9 knob	F19 knob	Ad9 antiserum	Ad19 antiserum
F9/F19 Ch2	14	5	1:8	1:128
F9/F19 Ch3	11	8	1:16	1:64
F9/F19 Ch4	9	10	1:64	1:32
F9/F19 Ch5	6	13	1:128	1:8
F9/F19 Ch6	4	15	1:128	1:8
F19/F9 Ch2	5	14	1:128	1:8
F19/F9 Ch3	8	11	1:64	1:16
F19/F9 Ch4	10	9	1:32	1:64
F19/F9 Ch5	13	6	1:8	1:128
F19/F9 Ch6	15	4	1:8	1:128
F9 MDis2.1	3	16	1:128	1:8
F9 MDis7.2	3	16	1:128	1:8
F9 MDis10.2	3	16	1:128	1:8
F9 MDis12.2	3	16	1:128	1:8
F9 MDis2	5	14	1:128	1:8
F9 MDis3	6	13	1:128	1:8
F9 MDis4	7	12	1:64	1:16
F9 MDis5	8	11	1:64	1:16
F9 MDis6	10	9	1:32	1:64
F9 MDis7	11	8	1:16	1:64
F9 MDis8	12	7	1:16	1:64
F9 MDis9	13	6	1:8	1:128
F9 MDis10	15	4	1:8	1:128

[a] The reactions were performed with human erythrocytes.
[b] All amino acid exchanges were between biochemically distinct groups.
[c] HI was detected in all cases.

tween biochemically distinct groups had the same effect. We therefore created the F9 MDis7.2, F9 MDis10.2, and F9 MDis12.2 fiber proteins. As predicted, they also showed a cross-reaction (Table 2).

Our results clearly showed that exchanging more than two of any of the 19 amino acids between biochemically distinct groups (which changes the charges and/or polarities of the corresponding sites) in the Ad9 and Ad19 fiber knob domains resulted in HI cross-reactivity with antisera against Ad9 and Ad19. Thirteen of the amino acids contributing to the Ad9 and Ad19 γ-determinants were distributed on five fiber knob loops (Fig. 4), with a concentration of 5 amino acids on loop CD (28, 29). The other six amino acids were situated in the D, F, H, and I β-sheet strands (Fig. 4). The 10 amino acids from biochemically identical groups did not contribute to the type-specific γ-determinants. While all chimeric or mutated fiber proteins with three to six amino exchanges between biochemically distinct groups showed the same HI titer as the corresponding unmutated fiber (1:128) and only a slight cross-reaction in HI tests (1:8), fibers with gradually more amino exchanges showed increased HI cross-reactions (Table 2). Our results indicate that the more type-specific antibodies are able to bind to the fiber knob (depending on the number of recognizable epitopes), the more strongly the binding of erythrocytes is inhibited.

DISCUSSION

The aim of this study was the molecular characterization of the type-specific γ-determinant on the adenovirus fiber. Although type-specific epitopes on the Ad2 fibers have been identified with monoclonal antibodies (25), the precise location on the fiber has so far not been determined. The type-specific γ-determinant can be demonstrated in HI tests. The ability of all human adenovirus serotypes to agglutinate erythrocytes (1, 20) was the basis for developing the HI test. Since the HI test is an important serological test which is applied, together with neutralization tests (S_n), for typing a given adenovirus serotype, it is important to gain more information about the amino acids involved in antigen-antibody binding. Ad9 and Ad19 showed no cross-reaction in HI tests, which clearly indicates that they possess distinct γ-determinants. Since the Ad9 and Ad19 knobs showed a total of 29 amino acid exchanges in the knob regions, we initially constructed, in order to confine the potential type-specific domains, seven chimeric Ad9/Ad19 fiber proteins (F9/F19 Ch1 to Ch7) and seven chimeric Ad19/Ad9 fiber proteins (F19/F9 Ch1 to Ch7). The first HI test results obtained with the chimeric fibers already indicated that the γ-determinant is not restricted to one area on the fiber knob. This is in contrast to the HA domain of subgenus B:2 adenoviruses, which is restricted to the HI loop of the fiber knob (12). In order to exactly determine the amino acids participating in the HI reaction, we created proteins with distinct amino acid exchanges. After having exchanged more than two amino acids between biochemically distinct groups, we obtained a cross-reaction in HI testing. Although the γ-determinant is composed of 19 amino acids in the case of Ad9 and Ad19, this is not an absolute number. The amount of amino acid residues contributing to the γ-antigen will vary between different serotypes depending on the homology of the fiber knobs. However, our data strongly suggest that all fiber knobs showing less than 17 amino acid exchanges between biochemically distinct groups will cross-react in HI tests. The 10 amino acid from biochemically identical groups did not contribute to the type-specific γ-determinants. But since amino acid exchanges between identical groups do not change the charges and/or polarities of the corresponding sites it is not surprising that such exchanges did not affect the γ-determinant and thus the antibody binding in HI tests.

Our findings are supported by sequence comparisons between the Ad8 and Ad9 fiber knobs (17). In the fiber knobs of Ad8 and Ad9 only nine exchanged amino acids (five amino acids from biochemically distinct groups) were found, and as expected both serotypes cross-reacted in HI tests (27). Although the Ad12 and Ad31 fibers exhibited a difference of 32 amino acids in the knob domains (18), they showed HI cross-reaction. At first, this seemed contradictory to our results, but subsequent amino acid analysis revealed that only 15 of these amino acids were from biochemically distinct amino acid groups, while the remaining 17 amino acids were from biochemically identical groups. These data correlated very well with our results and strongly supported our finding that amino acid exchanges within biochemically identical groups do not contribute to the γ-determinant.

The fiber knob is composed of β-strands, loops, and turns (28, 29). Since the loops contribute to the surface of the knob and are hence most accessible to antibodies, it could be expected that most of the amino acids contributing to the Ad9 and Ad19 γ-determinants would be located on these loops. Six amino acid exchanges in the β-strands could alter the conformation of the knob domain and thus could also alter the epitopes on the knob surface. Since Ad9 and Ad19 (and, as demonstrated, all our expressed fiber proteins) agglutinate rat and human erythrocytes with a complete pattern, they must possess identical HA domains. Although the precise mechanism by which HA is inhibited by type-specific antibodies remains unclear, our results suggest that the antibodies binding to the γ-determinant sterically inhibit the agglutination of erythrocytes. This assumption is supported by the results of Mei and Wadell (12), who characterized the HA domains of subgenus B:2 adenoviruses. Since these HA properties are

situated on the HI fiber loops, the HA domain(s) responsible for agglutinating rat and human erythrocytes could also be located on the loops and hence could be concealed by antibodies binding to the "loop domains" of the γ-determinant.

ACKNOWLEDGMENT

We thank T. Adrian at the National Reference Center for Adenoviruses in Hannover for kindly providing Ad9 and Ad19 antisera.

REFERENCES

1. **Bauer, H., and R. Wigand.** 1963. Eigenschaften der Adenovirus-Hämagglutinine. Z. Hyg. Infektionskr. **149:**96.
2. **Defer, C., M.-T. Belin, M.-L. Caillet-Boudin, and P. Boulanger.** 1990. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. J. Virol. **64:**3661–3673.
3. **Eiz, B., T. Adrian, and P. Pring-Åkerblom.** 1997. Recombinant fiber proteins of human adenoviruses Ad9, Ad15, and Ad19: localization of the haemagglutination and the type-specific determinant. Res. Virol. **148:**5–10.
4. **Green, M., N. G. Wrigley, W. C. Russell, S. R. Martin, and A. D. McLachlan.** 1983. Evidence for a cross-β-sheet structure in the adenovirus fibre. EMBO J. **2:**1357–1365.
5. **Henry, L. J., D. Xia, M. E. Wilke, J. Deisenhofer, and R. D. Gerard.** 1994. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. J. Virol. **68:**5239–5246.
6. **Hierholzer, J. C., and W. R. Dowdle.** 1970. Immunological basis of the adenovirus 8-9 cross-reaction. J. Virol. **6:**782–787.
7. **Higuchi, R.** 1991. Recombinant PCR, p. 177–183. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.) PCR protocols. A guide to methods and applications. Academic Press, Inc., San Diego, Calif.
8. **Hong, J. S., and J. A. Engler.** 1991. The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal. Virology **185:**758–767.
9. **Horne, R. W., S. Brenner, A. P. Waterson, and P. Wildy.** 1959. The icosahedral form of an adenovirus. J. Mol. Biol. **1:**84–86.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
11. **Louis, N., P. Fender, A. Barge, P. Kitts, and J. Chroboczek.** 1994. Cell-binding domain of adenovirus serotype 2 fiber. J. Virol. **68:**4104–4106.
12. **Mei, Y.-F., and G. Wadell.** 1996. Epitopes and hemagglutination binding domain on subgenus B:2 adenovirus fibers. J. Virol. **70:**3688–3697.
13. **Norrby, E.** 1968. Biological significance of structural adenovirus components. Curr. Top. Microbiol. Immunol. **43:**1.
14. **Norrby, E.** 1969. The structural and functional diversity of adenovirus capsid components. J. Gen. Virol. **5:**221–236.
15. **Novelli, A., and P. Boulanger.** 1991. Deletion analysis of functional domains in baculovirus-expressed adenovirus type 2 fiber. Virology **185:**365–376.
16. **Philipson, L., K. Lonberg-Holm, and U. Pettersson.** 1968. Virus-receptor interaction in an adenovirus system. J. Virol. **2:**1064–1075.
17. **Pring-Åkerblom, P., and T. Adrian.** 1995. Characterization of adenovirus subgenus D fiber genes. Virology **206:**564–571.
18. **Pring-Åkerblom, P., and T. Adrian.** 1995. Sequence characterization of the adenovirus 31 fibre and comparison with serotypes of subgenera A to F. Res. Virol. **146:**343–354.
19. **Pring-Åkerblom, P., A. Heim, and F. E. J. Trijssennaar.** 1997. Conserved sequences in the fibers of epidemic keratoconjunctivitis associated human adenoviruses. Arch. Virol. **142:**205–211.
20. **Rosen, L.** 1960. A hemagglutination-inhibition technique for typing adenoviruses. Am. J. Hyg. **71:**120–128.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
22. **Signäs, C., G. Akusjärvi, and U. Pettersson.** 1985. Adenovirus 3 fiber polypeptide gene: implications for the structure of the fiber protein. J. Virol. **53:**672–678.
23. **Stevenson, S. C., M. Rollence, B. White, L. Weaver, and A. McClelland.** 1995. Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. J. Virol. **69:**2850–2857.
24. **Stouten, P. F. W., and C. Sander.** 1992. New triple-helical model for the shaft of the adenovirus fibre. J. Mol. Biol. **226:**1073–1084.
25. **Watson, G., M. G. Burdon, and W. C. Russell.** 1988. An antigenic analysis of the adenovirus type 2 fibre polypeptide. J. Gen. Virol. **69:**525–535.
26. **Wigand, R.** 1970. Adenoviruses types 20, 25, and 28: atypical members of group II. J. Gen. Virol. **6:**325–328.
27. **Wigand, R.** 1987. Pitfalls in the identification of adenoviruses. J. Virol. Methods **16:**161–169.
28. **Xia, D., L. J. Henry, R. D. Gerard, and J. Deisenhofer.** 1994. Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution. Structure (London) **2:**309–319.
29. **Xia, D., L. Henry, R. D. Gerard, and J. Deisenhofer.** 1995. Structure of the receptor binding domain of adenovirus type 5 fiber protein, p. 39–46. *In* W. Doerfler and P. Boehm (ed.), The molecular repertoire of adenoviruses I. Springer-Verlag, Berlin, Germany.