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Biochemical and genetical characterization of a fiber-defective temperature-sensitive mutant of type 2 adenovirus

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The adenovirus type 2 fiber mutant H2 *ts* 125 synthesized an unstable, temperature-sensitive fiber polypeptide with an apparent mol. wt. smaller by 2500 than the wild-type (62 K). The polypeptide of 59.5 K was found to be stable at the permissive temperature (33°C). H2 *ts* 125 fiber synthesized in reticulocyte lysates had the same apparent mol. wt. of 59.5 K as the mutant fiber produced *in vivo*. Neither structural nor functional differences between wild-type and mutant fibers were detected in the N-terminal and C-terminal sequences, excluding the occurrence of a new initiation or termination codon. Restriction analysis of H2 *ts* 125 DNA also ruled out the hypothesis of a deletion mutant. The 59.5 K mutant fiber unit was normally glycosyated, N-acetylated, assembled into 6S oligomeric fiber and incorporated into virions. DNA sequencing of the H2 *ts* 125 fiber gene revealed two point mutations at nucleotides 3970 (C*TT→T*TT) and 4958 (GC*T→GT*T), corresponding to two amino acid changes at positions 105 and 434, respectively. The 105 mutation consisted of a conservative change Leu→Phe; the 434 interchange was Ala→Val, usually considered as non-conservative. Th possibility of a donor site for splicing created by the mutation at codon GTT was eliminated on the basis of S1 nuclease analysis data. All these results suggested that either one or both mutations concerned highly organized domain(s) of the fiber polypeptide chain, resulting in aberrant mobility in SDS-polyacrylamide gels and temperature-sensitivity.

Key words: adenovirus 2/fiber protein/DNA sequence/fiber gene/*ts* mutants

Introduction

Sets of temperature-sensitive (*ts*) mutants of adenoviruses have been selected in several laboratories to determine the role of adenovirus gene products involved in viral replication and morphogenesis, as well as to study various aspects of the virus-cell interactions (reviewed by Ginsberg and Young, 1977). The adenovirus 2 mutant H2 *ts* 125 has been isolated in our laboratory after mutagenization of a wild-type (WT) stock, and partially characterized (Martin *et al.*, 1975). It is altered in the production of antigenically reactive fiber and accumulates light assembly intermediate particles (or top components) at the non-permissive temperature (D'Halluin *et al.*, 1980: Martin *et al.*, 1978).

H2 *ts* 125 has been recently mapped between coordinates 81 and 100 on the genomic map (D'Halluin *et al.*, 1982), a position which overlaps the fiber gene (85.5 – 91.8 map units) (Miller *et al.*, 1980). It has been found to be altered in the fiber structure: the fiber polypeptide unit (polypeptide IV) is of lower apparent mol. wt. in SDS-polyacrylamide gel than the WT fiber polypeptide unit (D'Halluin *et al.*, 1980). A further biochemical characterization of the H2 *ts* 125 is presented here, and the genetical lesion determined by DNA sequencing of the mutant fiber gene.

Results

Synthesis, post-translational modifications and virion incorporation of fiber in H2 ts 125 mutant

Synthesis of fiber polypeptide in H2 ts 125 infected-cells and virion incorporation. KB cells were infected with WT or H2 *ts* 125 at 10 f.f.u./cell at 39.5°C and pulse-labeled for 1 h at 16 h after infection. The cell culture was then divided into two portions, one being harvested just after the pulse, the other one being chased for 8 h at 39.5°C. Virus-coded polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Figure 1A shows that in the H2 *ts* 125 pattern at 39.5°C, the apparent mol. wt. of the fiber polypeptide (IV) was 2500 lower than that in the WT. Careful determination by electrophoresis in 30-cm slab gels with mol. wt. standards (Pharmacia LMW calibration kit) gave the value of 59 500 for H2 *ts* 125 fiber, instead of 62 000 for WT fiber (theoretical value: 61 882; Hérissé *et al.*, 1981). The label in

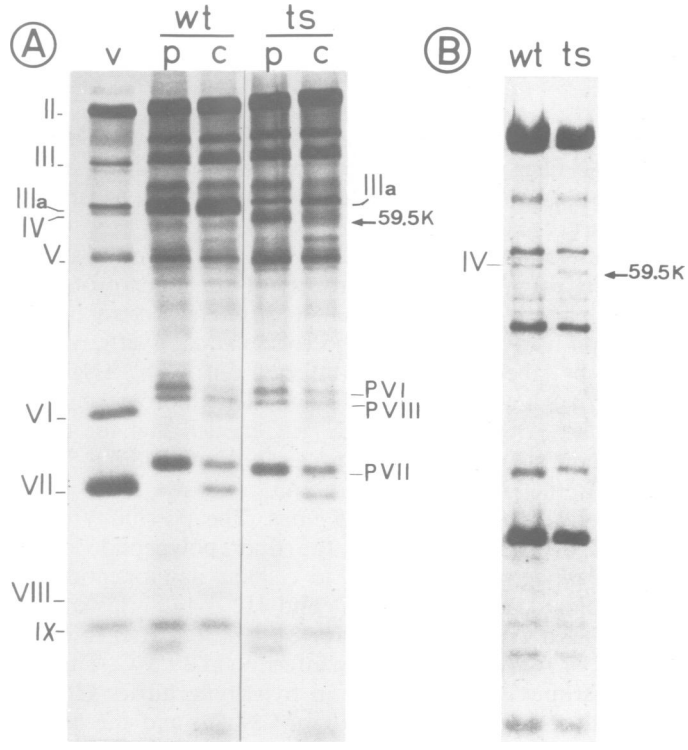

Fig. 1. SDS-polyacrylamide gel analysis of WT and mutant fiber polypeptides in cells and virions. **(A)** Pulse-chase experiments at 39.5°C. The cells were labeled with [^{35}S]methionine for 1 h at 16 h after infection. (p) 1 h pulse, (c) 8 h chase. **(B)** Stained gel (Coomassie blue) showing WT and H2 *ts* 125 virus particles produced at the permissive temperature. In cells as well as in virion, the fiber polypeptide (IV) has an apparent mol. wt. of 62 000 in WT and 59 500 in H2 *ts* 125.

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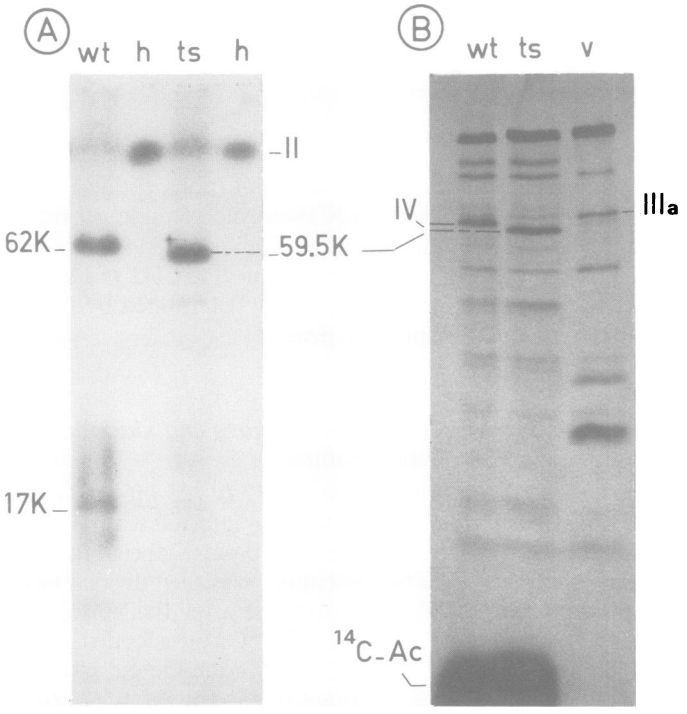

Fig. 2. SDS-polyacrylamide gel analysis of the post-translational modifications of the WT and mutant fibers. **(A)** Glycosylation. The WT-and H2 *ts* 125-infected cells were labeled with [^{3}H]glucosamine for 8 h at 16 h after infection. (v): control [^{3}H]leucine-labeled virion; (h): [^{3}H]leucine-labeled hexon marker; (wt): [^{3}H]glucosamine-labeled wild-type extract; (ts): [^{3}H]glucosamine-labeled H2 *ts* 125-mutant extract. **(B)** N-acetylation. WT- and H2 *ts* 125-infected cells were labeled with [^{14}C]sodium acetate for 24 h at 56 h after infection at 33°C. (v): Control [^{14}C]valine-labeled virion; (wt): WT-extract; (ts): H2 *ts* 125 extract. **Gels A** and **B** were revealed by fluorography at −70°C. the mutant fiber polypeptide migrated with an apparent mol. wt. of 59.5 K, instead of 62 K for the WT.

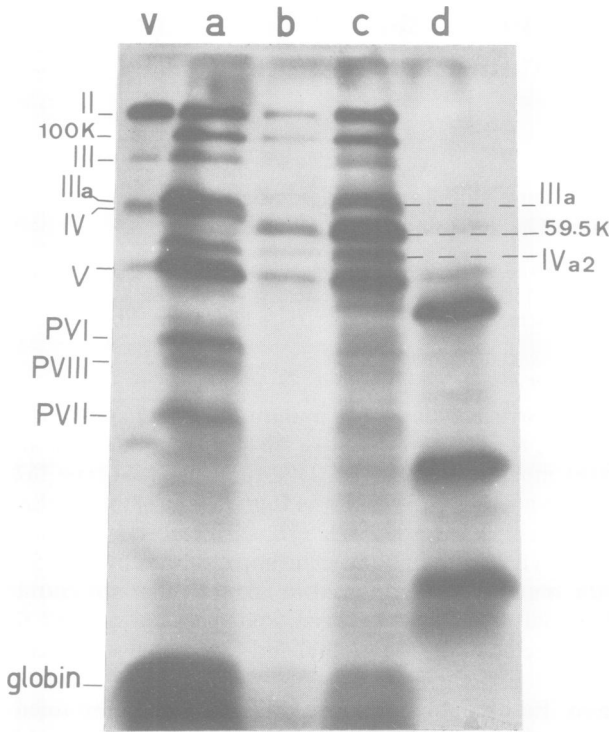

Fig. 3. Cell-free translation of WT and H2 *ts* 125 late mRNAs. (v): control [^{14}C]valine-labeled virion polypeptide markers. **(a)** WT mRNAs translated *in vitro* in the presence of 2 mM $MgCl_2$, 130 mM KCl; **(b)** H2 *ts* 125 mRNAs with 3 mM $MgCl_2$, 13 mM KCl; **(c)** H2 *ts* 125 mRNAs translated as in **(a)**; **(d)** parotid gland collagen mRNAs used as control.

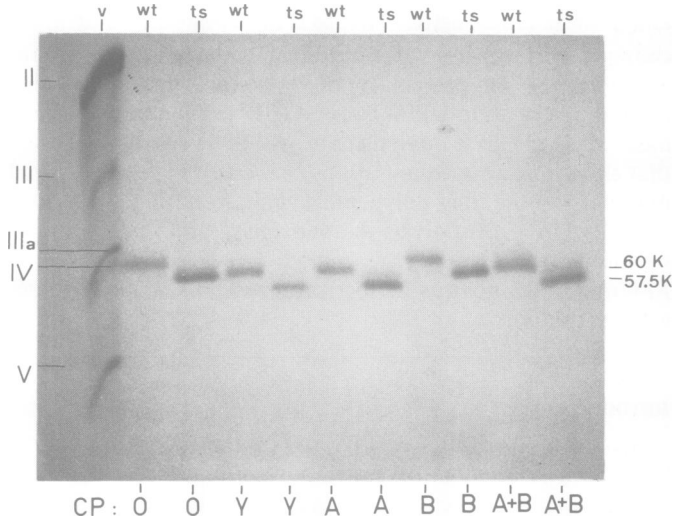

Fig. 4. Comparative carboxypeptidase digestions of WT and H2 *ts* 125 fibers. Samples of purified [^{35}S]methionine-labeled fiber were incubated with carboxypeptidase (CP) A (A), carboxypeptidase B (B), a mixture of both carboxypeptidases A and B (A + B), carboxypeptidase Y (Y), or no carboxypeptidase (0) and analyzed in SDS-gel. (wt): wild-type fiber; (ts): mutant fiber. The same type of carboxypeptidase-resistant core was obtained with single CPA, CPY or double CPA + CPB digestion: 60 K and 57.5 K for WT and mutant fibers, respectively. Both types of fibers seemed insensitive to CPB action. v: control virion.

59.5 K polypeptide disappeared during the chase at 39.5°C. A shorter fiber polypeptide unit was also found incorporated in the H2 *ts* 125 virus particles assembled at the permissive temperature (Figure 1B).

Glycosylation of H2 ts 125 fiber. The hypothesis of a defect in the glycosylation process of the fiber protein at 39.5°C, which could result in a different electrophoretic migration (Leavitt *et al.*, 1977; Persson *et al.*, 1980), was examined by labeling H2 *ts* 125-infected cells with [^{3}H]glucosamine for 8 h at 16 h after infection. The fluorographic pattern of an SDS-polyacrylamide gel revealed that the major late glycoprotein was a 62 K species in the WT and a 59.5 K species in the H2 *ts* 125 (Figure 2A).

N-acetylation of H2 ts 125 fiber. The possibility of an absence of N-acetylation of the fiber polypeptide chain, thereby rendering it sensitive to cellular aminopeptidase(s) was explored as follows. WT- and H2 *ts* 125-infected cells were labeled with [^{14}C]acetate for 24 h at 56 h after infection at 33°C or for 8 h at 16 h after infection at 39.5°C. The cells were disrupted by sonication in hypotonic buffer (10 mM Tris-hydrochloride, pH 7.5, 50 mM NaCl) and the cell extract centrifuged at 10 000 *g* for 60 min. The supernatant was analyzed by SDS-PAGE. As shown in Figure 2B, most of the primary translational products of WT adenovirus were N-acetylated: II, 100 K, III and IV appeared as the major ^{14}C-acetylated adenovirus polypeptides; minor bands of [^{14}C]-acetate-labeled IIIa, V, 39 K, pVI and pVIII were also visible. In H2 *ts* 125-infected cell extract, a major species of ^{14}C-N-acetylated 59.5 K was observed in place of the 62 K fiber polypeptide unit present in the WT, at either 39.5° or 33°C. The H2 *ts* 125 polypeptide unit of 59 500 daltons was therefore normally N-acetylated. The H2 *ts* 125 fiber also showed the same resistance to aminopeptidase M digestion as

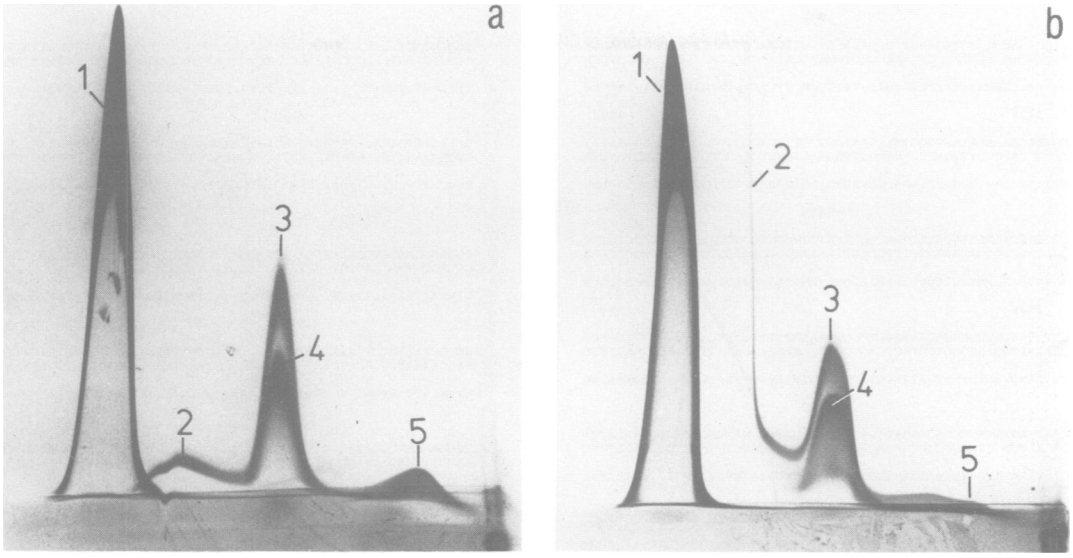

Fig. 5. Assembly of penton base and mutant fiber *in vitro*. 2D pattern of H2 *ts* 125-infected cell extract maintained at 33°C and analyzed before (**a**) and after (**b**) addition of a large excess of unlabeled penton base. The significant decrease of the peak of free fiber (peak 5), suggested an *in vitro* binding with penton base to form penton (peak 3). Note the simultaneous increase of penton base peak and the dilution of label in cold penton base (peak 2).

the WT fiber (not shown). This suggested that the 59.5 K molecule did not derive from the 62 K unit by cleavage at the N terminus.

Assembly of H2 ts 125 fiber polypeptide chains into 6S oligomer

Sedimentation analyses of H2 *ts* 125-infected cell extracts were carried out to evaluate the capacity of fiber polypeptide chains to assemble into 6S fiber capsomers. Fiber polypeptide units of 59.5 K, synthesized at 39.5°C, were found in the 10–11S region of the gradient, as penton base-attached fibers, and in the 6S domain co-sedimenting with *Escherichia coli* alkaline phosphatase (6.0S). Immunoselection on *Staphylococcus aureus* protein A or immunodetection by Western blotting of fiber antigenic determinants confirmed the presence of antigenic fiber in the 10.5S and 6S regions of the gradient, but also in the 3.0–3.5S zone, where the free fiber polypeptide chains sedimented (not shown). These results suggested that the H2 *ts* 125 made 59 500 mol. wt. fiber polypeptides capable of self-assembling at the non-permissive temperature to form 6S oligomeric fibers.

In vitro synthesis and N-terminal sequence of mutant fiber

Total cytoplasmic RNA, obtained from WT- or H2 *ts* 125-infected cells, at 20 h after infection, was translated *in vitro* in reticulocyte lysate, and the products acid-precipitated. As shown in Figure 3, the cell-free translated mutant fiber behaved as a 59.5 K polypeptide, as did the H2 *ts* 125 fiber unit synthesized *in vivo*. This seemed to exclude a change in post-translational modification of the mutant fiber as a hypothesis to explain its lower apparent mol. wt.

The [^{3}H]proline-labeled WT and mutant fibers were purified by electroelution from an SDS-gel (Anderson and Lewis, 1980) and sequenced in an automatic sequencer. Proline was recovered at positions 6, 13 and 16 for both fibers, as predicted from the sequence of WT DNA (Hérissé and Galibert, 1981). This confirmed the structural identity of WT and mutant fiber N termini.

Structural analysis of the C terminus of the H2 ts 125 fiber

[^{35}S]Methionine-labeled WT and H2 *ts* 125 fibers purified as previously described were digested with three different carboxypeptidases, A, B and Y, at a ratio of enzyme to substrate of 5:100 for 24 h at 37°C, and enzymic cleavage products analyzed by SDS-PAGE. Carboxypeptidase Y (CP-Y) removed 2000 daltons from the C end of both fibers, corresponding to ~20 amino acids: the WT fiber of 62 K was cleaved to a CP-Y-resistant core of 60 K, and the H2 *ts* 125 fiber (59.5 K) to a 57.5 K core. The same type of cleavage was obtained with CP-A, wheras both WT and H2 *ts* 125 fibers proved insensitive to CP-B action (Figure 4). Pepstatin A-treated CP-Y (Lee and Riordan, 1978) cleaved off the same amino acid chain length from both fibers as did untreated CP-Y, which seemed to exclude a cleavage by contaminating endopeptidases. In addition, SDS-denatured fibers from WT and H2 *ts* 125 showed the same pattern of CP-A digestion as native fibers (not shown). This also suggested that cleavage did occur at the C end and was limited by the first basic residue (a lysine) at position 19 upstream from the carboxyl terminus (Hérissé *et al.*, 1981). A mixture of CP-A and CP-B resulted in the same CP-resistant cores as with CP-A alone. All these results were compatible with the published amino acid sequence of adenovirus 2 fiber carboxylic region (Hérissé *et al.*, 1981).

The limited digestions of native fibers obtained with a mixture of CP-A and CP-B, as well as with CP-Y, was probably due to some secondary structure of the fiber protein, rather than to the presence of a particular amino acid residue. This hypothesis was supported by the fact that WT fiber was totally digested by CP-Y after SDS denaturation (not shown). The identity of WT and H2 *ts* 125 carboxypeptidase-resistant cores suggested therefore a structural identity of the C-terminal end between both types of fiber. This was confirmed by the determination of the amino acids released from the C terminus by carboxypeptidase.

WT and H2 *ts* 125 fibers were labeled in infected cells with a mixture of [^{14}C]amino acids and subjected to CP-Y digestion. The fiber core and residual enzyme were precipitated with 10% formic acid and the acidic supernatant dried in a vacuum dessicator. The dry residue was dissolved in 10% pyridine and analyzed by two-dimensional (2D) chromato-

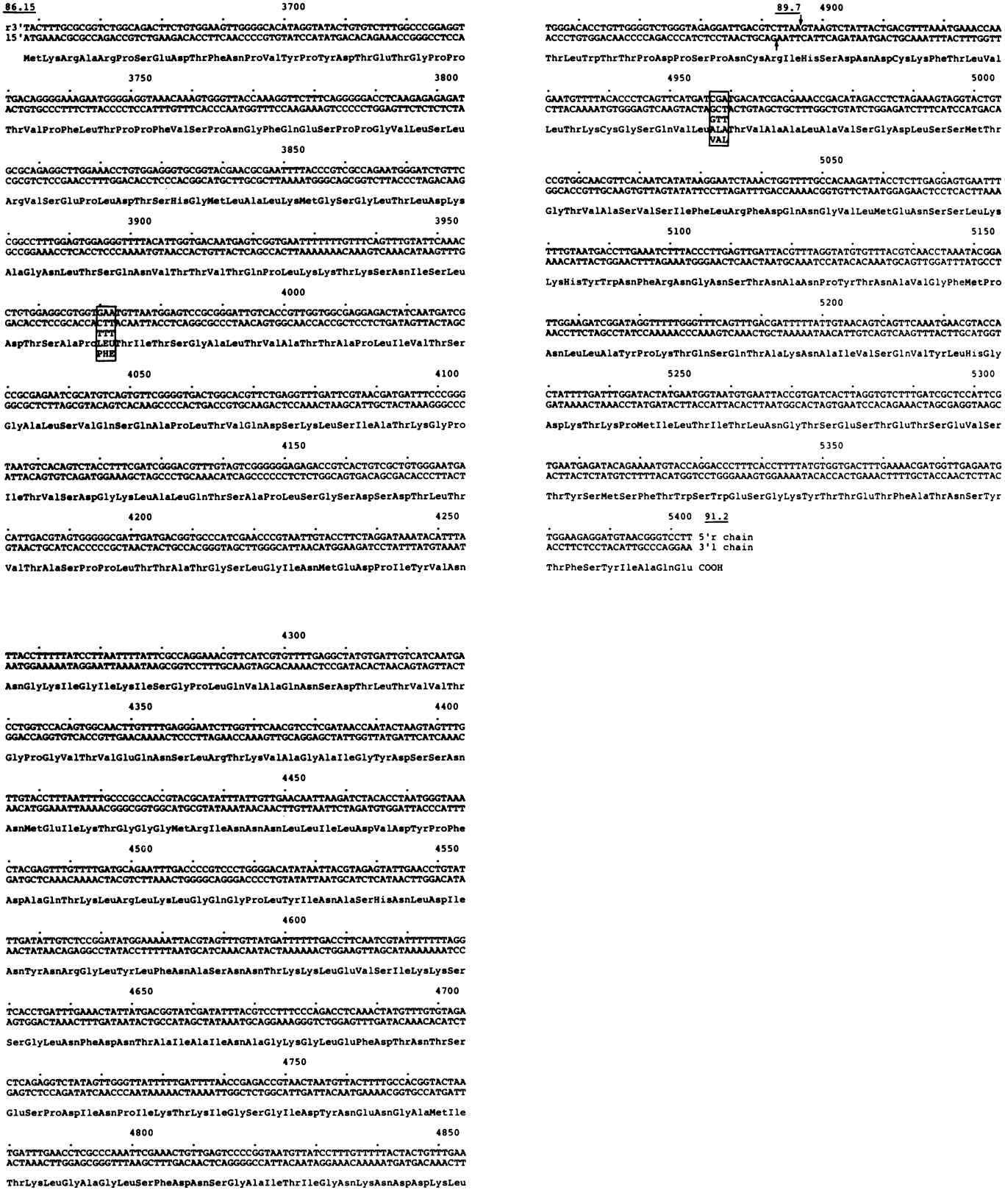

Fig. 6. Nucleotide sequence of adenovirus 2 fiber gene and sites of mutation of H2 *ts* 125. The mutated codons are framed. The arrows indicates the cleavage site for *Eco*RI at map position 89.7. Map coordinates for the fiber gene are underlined. Nucleotides are numbered according to Hérissé *et al.* (1981).

graphy. The 2D patterns were found to be identical for WT and H2 *ts* 125 fibers, with four major spots of tyrosine, threonine, glutamic acid and isoleucine (not shown).

Functional integrity of the C-terminal sequence of the H2 ts 125 fiber

It has been recently shown that the attachment of the fiber with the penton base involves the C-terminal end of the fiber, and that this C end lies opposite the distal knob (Boudin and Boulanger, 1982). In the light of this, if the H2 *ts* 125 fiber was capable of assembly with the penton base *in vitro* and/or *in vivo*, this would imply a functional integrity of the C-terminal end of the mutant fiber. The presence of penton in extracts of cells infected with H2 *ts* 125 at 33°C, as evidenced by immunological analyses and by electron microscopy (not shown) as well as in H2 *ts* 125 virion (Figure 1B), indicated that the assembly of H2 *ts* 125 penton base and fiber could occur *in vivo*. The asembly could also occur *in vitro*, as shown in Figure 5. When [^{35}S]methionine-labeled extracts of cells infected with the mutant at 33°C were incubated overnight at 4°C (Boudin and Boulanger, 1982) with a large excess of cold H2 *ts* 125 penton base isolated as previously described (Boudin *et al.*, 1979), the peak of free fiber decreased, as a result of its binding with penton base to form penton (Boudin and Boulanger, 1982). This suggested that the H2 *ts* 125 mutation did not alter the biological function of the fiber C end.

DNA sequencing of the H2 ts 125 fiber gene

All the data reported above suggested, therefore, that point mutation(s) were responsible for aberrant mobility of H2 *ts* 125 fiber in SDS-PAGE, and also plausibly temperature-sensitivity. The two halves of the H2 *ts* 125 fiber gene, corresponding to fragments *Eco*RI-E and *Hind*III-F were cloned and sequenced according to the same strategy as that used for the WT fiber gene (Hérissé and Galibert, 1981; Hérissé *et al.*, 1981).

The sequence derived from the H2 *ts* 125 fiber mutant was identical to that of the WT except for two point mutations. At position 3970, on the sense strand, a C residue was replaced by a T, changing the CTT triplet to a TTT triplet. At position 4958, a C residue was also replaced by a T residue changing the previous GCT triplet to GTT. A detailed map indicating the sites of mutation is presented in Figure 6.

S1 endonuclease analysis of H2 ts 125 fiber message

Since the mutation at nucleotide 4958 could create a donor site for a splice, comparative S1 analysis of WT and H2 *ts* 125 fiber mRNAs was designed to test this hypothesis. Hybridization was performed with short fragments containing either one or the other point mutations, but also with longer genomic fragments corresponding to either the left-hand or right-hand moiety of the fiber gene. No difference between WT and H2 *ts* 125 S1 patterns was observed, suggesting that no splicing occurred in the mutant fiber.

Discussion

A temperature-sensitive, fiber-defective mutant of type 2 adenovirus, H2 *ts* 125, has been isolated and phenotypically characterized (D'Halluin *et al.*, 1980; Martin *et al.*, 1978). It maps in the fiber gene (D'Halluin *et al.*, 1982). Further biochemical characterization showed that the mutant-infected cells made a fiber polypeptide unstable at the non-permissive temperature (39.5°C), and smaller by a mol. wt. of 2500 than that of the WT, *viz.*, 59.5 K instead of 62 K. The 59.5 K fiber polypeptide also occurred at the permissive temperature (33°C), but appeared stable at this temperature (Figure 1).

Several hypotheses can be formulated to explain the biochemical properties of the fiber mutant. (i) A mutation on the amino end might result in the lack of acetylation of the N-terminal amino acid of the polypeptide chain, thereby rendering it sensitive to cellular aminopeptidase(s). (ii) A mutation lying somewhere within the amino acid sequence might reveal preferred site(s) for cellular endopeptidase(s) cleavage, located at 20 amino acids either from the amino or carboxy end. (iii) The H2 *ts* 125 mutation might change the nucleotide sequence to a stop codon, leading to a shorter polypeptide chain. (iv) Alternatively, a mutation could suppress the normal initiation codon and create a new initiation codon at ~60 nucleotides downstream. (v) Point mutation(s) might provoke a change in the three-dimensional structure of the mutant fiber which affects its migration in SDS-gel and its morphology under the electron microscope. (vi) The mutant H2 *ts* 125 might be a deletion mutant originally present in the mutagenized WT stock, and selected for its temperature-sensitive phenotype. (vii) Point mutation might create a new acceptor or donor splice site used in conjunction with other consensus sequences provoking the occurrence of a short intron.

The data presented here ruled out a number of these alternatives. The mutant fiber polypeptide was normally glycosylated, acetylated and assembled into 6S oligomeric, antigenically reactive fiber. In pulse-labeling experiments, a fiber polypeptide unit of 62 K was never found in H2 *ts* 125-infected cells, which seemed to exclude that the 59.5 K polypeptide might derive from a 62 K species by proteolytic cleavage. The H2 *ts* 125 fiber was also capable of assembly *in vivo* and *in vitro* with penton base to form penton. This suggested a functional integrity of the C-terminal sequence of the mutant fiber, since the C end of the fiber has been found to be involved in the linkage with the penton base unit (Boudin and Boulanger, 1982). However, recent structural data are consistent with a model of attachment of fiber with penton base via its N-terminus (Green *et al.*, 1983). Nevertheless, biochemical analyses revealed that the N and C termini of the mutant fiber were undistinguishable from those of the WT fiber, excluding the four first hypotheses. Restriction analysis of H2 *ts* 125 DNA also excluded a deletion within the fiber gene.

Since an unambiguous answer might only be furnished by the nucleotide sequence of the H2 *ts* 125 fiber genome, *Eco*RI-E and *Hind*III-F fragments from H2 *ts* 125 DNA, overlapping the gene for fiber, were cloned and sequenced. Two point mutations were found at nucleotides 3970 and 4958, corresponding to two amino acid changes at positions 105 and 434, downstream from the N end. The first change was of a conservative type (Leu→Phe), whereas the second one consisted of a non-conservative interchange Ala→Val (Dayhoff, 1969). The codon change GC*T→GT*T might create a donor site for splicing, according to the splice junction consenses sequences (Mount, 1982). A spliced sequence within the fiber gene might explain a shorter fiber polypeptide unit. However, the S1 analysis data showed no evidence for the existence of a splice within the fiber gene.

Taken altogether, these data suggested that either one or both point mutations concerned highly organized domain(s) of the fiber polypeptide chain, having a major influence on its

electrophoretic mobility and its temperature stability. Both WT and mutant fiber protein sequences are now being computerized to determine the possible conformational changes resulting from either one or the other mutation. In addition, to elucidate the mechanism of the temperature-sensitivity of adenovirus mutant fiber, antibodies are being prepared against synthetic peptides reproducing the WT and mutated amino acid sequences.

Materials and methods

Cell cultures and viruses

Suspension cultures of KB cells were grown in Eagle spinner medium supplemented with 5% horse serum. HeLa cells were cultured as monolayers in Dulbecco-modified Eagle medium supplemented with 10% calf serum. H2 temperature-sensitive mutant H2 *ts* 125 was isolated in our laboratory after nitrous acid treatment of a WT stock (Martin *et al.*, 1978). Stocks of *ts* mutants were grown on HeLa cell monolayers maintained at 33°C. Adenovirus was titrated by the plaque assay or by the fluorescent focus units (f.f.u.) assay (Philipson *et al.*, 1968) on a HeLa cell monolayer at 37°C. The cells were usually infected at a multiplicity of infection of 10–100 f.f.u./cell.

Purification of virus and viral DNA

Adenovirus particles produced at 37°C by the WT or at 33°C by the H2 *ts* 125 were extracted from infected cells with Freon 113 and purified in self-generating CsCl gradients, as previously described (D'Halluin *et al.*, 1978). Viral DNA was isolated as a DNA-terminal protein complex. Virus particles were disrupted by adding an equal volume of 8 M guanidinium chloride made in 0.2 M Tris-hydrochloride, pH 8.1, 20 mM Na EDTA, 4 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) for 10 min in the cold. The virus lysate was then chromatographed on a Sepharose 4 B (Pharmacia Fine Chemicals) column (60 x 1.5 cm) equilibrated in 0.1 M Tris-hydrochloride, pH 8.1, 10 mM Na EDTA, 2 mM 2-mercaptoethanol. The excluded peak was adjusted to 3.06 M in CsCl and the DNA-terminal protein complex was banded in a Kontron TFT 65 fixed angle rotor for 72 h at 33 000 r.p.m. and 20°C.

Labeling conditions

Virus polypeptides in infected cells. Pulse-chase labelings of virus-coded proteins were performed in KB cells (6 x 10^6 cells/ml) in methionine-deprived medium. L-[^{35}S]Methionine (50 μCi/ml) was added for 1 h at 16 h after infection at 39.5°C. The cells were either harvested just after the pulse, or after a chase of 8 h at 39.5°C. Label was chased by dilution to 3 x 10^5 cells/ml in medium prewarmed to the required temperature and containing 10 times the normal concentration of cold methionine.

Uniform labeling of the WT and mutant fibers. The adenovirus fiber was uniformly labeled *in vivo* by adding a mixture of [^{14}C]amino acids (10 μCi/ml) to a cell culture with an amino acid concentration 10 times lower than that of the normal medium. Labeling was performed for 24 h at 56 h after infection at 33°C, and the fiber purified as previously described (Boudin and Boulanger, 1982; Boulanger and Puvion, 1973). Labeling of carbohydrate units of adenovirus fiber was carried out by the addition of [^{3}H]glucosamine to the cell culture (50 μCi/ml) for 8 h at 16 h after infection at 39.5°C.

N-terminal labeling of adnenovirus fiber. Most of the primary translocation products of adenovirus have been shown to be acetylated (Jörnvall *et al.*, 1974). Fiber was labeled on its N terminus by addition of [^{14}C]sodium acetate (Fedor and Daniell, 1980) (10 μCi/ml) for 24 h at 56 h after infection at 33°C or for 8 h at 16 h after infection at 39.5°C. Labeled virus-coded proteins and glycoproteins were analyzed in SDS-containing polyacrylamide gels.

Analytical SDS-PAGE. Samples were dissolved in an equal volume of sample buffer (0.0625 M Tris-hydrochloride, pH 6.8, containing 6 M urea, 5% SDS, 10% mercaptoethanol and 0.005% bromophenol blue) and heated for 2 min at 100°C. Polypeptides were analyzed in an SDS-containing 15.5% polyacrylamide gel (acrylamide/bisacrylamide ratio of 50:0.235) overlaid by a 5% spacer gel (acrylamide/bisacrylamide ratio of 50:1.33) in the discontinuous buffer system of Laemmli (1970). The gels were stained with Coomassie brilliant blue (Serva blue, R.250), dried under vacuum and exposed to Kodak Royal X-OMAT S film. ^{3}H-labeled bands were revealed by fluorography at −70°C, after impregnating the gels with PPO in DMSO (Bonner and Laskey, 1974).

2D immunoelectrophoresis

2D analyses were carried out as described in detail elsewhere (Martin *et al.*, 1975, 1978).

Assembly of fiber capsomers

Antigenically reactive trimeric hexons sediment at 12–13S, pentons at 10.5S and penton base at 9.1S (Boudin *et al.*, 1979; Lemay and Boulanger, 1980; Pettersson and Höglund, 1969; Velicer and Ginsberg, 1970). Fiber capsomers sediment as 6.1S components (Lemay and Boulanger, 1980; Sundquist *et al.*, 1973; Wilhelm and Ginsberg, 1972), whereas unassembled fiber polypeptide chains sediment at ~3.4S (Velicer and Ginsberg, 1970). To evaluate the capacity of the fiber polypeptide chains to assemble into the 6S oligomeric form, H2 *ts* 125-infected HeLa cells were pulse-labeled with [^{35}S]methionine for 1 h at 18 h after infection. After three washes in Tris-saline, the cells were collected by centrifugation and resuspended in 0.5 ml of extraction buffer (20 mM Tris-hydrochloride, pH 7.5, 10 mM NaCl, 0.5% Triton X-100, 2 mM PMSF) and disrupted by sonication. NaCl was added up to 0.15 M final concentration and the extract was centrifuged for 1 h at 100 000 *g* and 4°C. The supernatant was brought to 1 M NaCl and analyzed by velocity sedimentation in a sucrose gradient. The gradients contained 5–27% sucrose (w:v) in 0.02 M Tris-hydrochloride, pH 8.1, 1 M NaCl, 10% (v:v) glycerol and 1 mM Na EDTA. The gradients were centrifuged for 21 h at 49 000 r.p.m. at 4°C in a Beckman SW 50.1 rotor (Lemay and Boulanger, 1980). Fractions (0.2 ml) were collected through the bottom of the centrifuge tube. The gradients were calibrated with beef liver catalase (11.2S), *E. coli* alkaline phosphatase (6.0S), human blood amylase (4.5S) and yeast 3-phosphoglycerate kinase (3.1S). Enzyme markers were assayed as previously described (Lemay and Boulanger, 1980), using a Rotochem II A 36 centrifugal analyzer (Roche-Kontron). Adenovirus 2 fiber was revealed in gradient fractions by immunoselection on *S. aureus* cells (Kessler, 1975), or by Western blotting (Towbin *et al.*, 1979), using an anti-fiber rabbit serum (Boudin and Boulanger, 1981).

Carboxypeptidase digestions

Samples of WT and H2 *ts* 125 fibers produced at 33°C and purified as previously described (Boulanger and Puvion, 1973) were digested with carboxypeptidase A, B or Y at a ratio of enzyme to substrate of 5:100 for 24 h at 37°C in 0.05 M sodium phosphate buffer pH 6.8. Hydrolyzates were analyzed in SDS-PAGE as described above. Amino acids cleaved off the C terminus were analyzed in the acid-soluble supernatant by 2D cellulose t.l.c. in the following solvent system. First dimension: 2-propanol/2-butanone/1 N-HCl (60/15/25). Second dimension: 2-methyl-2-butanol/2-butanone/acetone/methanol/0.885 M ammonia solution/H_2O (50/20/10/5/5/15).

In vitro synthesis and N-terminal sequence of H2 ts 125 fiber

Total cytoplasmic RNA was obtained from WT- and H2 *ts* 125-infected cells harvested at 20 h after infection at 37°C, according to Anderson *et al.* (1974), Anderson and Lewis (1980), and translated *in vitro* in reticulocyte lysate/[^{3}H]proline (New England Nuclear, Dreieich, FRG). K^+ and Mg^{2+} concentrations were brought to 150 mM and 2 mM, respectively. [^{3}H]-Proline-labeled products synthesized *in vitro* were analyzed in SDS-polyacrylamide gel and [^{3}H]proline-labeled fiber was purified by electroelution (Anderson and Lewis, 1980). Proline has been found at positions 6, 13 and 16 on the fiber N end (Anderson and Lewis, 1980; Hérissé and Galibert, 1981). The N terminus of [^{3}H]proline-labeled WT and mutant fibers was sequenced in a Beckman 890 C automatic sequencer, using apomyoglobin as carrier. The radioactivity of each cycle was determined in a liquid spectrometer.

Enzymes

Peptidases. Diisopropylfluorophosphate-treated carboxypeptidase A, from bovine pancreas, 2 x crystallized and carboxypeptidase B, from porcine pancreas, 2 x crystallized, were both purchased from Sigma. Yeast carboxypeptidase Y was purchased from Pierce Chemicals, and treated with pepstatin-A (Sigma) according to Lee and Riordan (1978). Aminopeptidase M, from hog kidney, was obtained from Boehringer.

Restriction endonucleases. *Eco*RI, *Sma*I, *Hind*III and *Bgl*II and *Sph*I (D'Halluin *et al.*, 1983) were purchased from Boehringer; *Xba*I from Miles Laboratories; *Hha*I from BRL, and *Hinf*I from Amersham. All reaction mixtures for restriction cleavage contained 1 μg of viral DNA. Each reaction buffer was made according to the manufacturer's recommendations. Viral DNA was cleaved as DNA-terminal protein complex, and pronase (Calbiochem) added for 15 min at 37°C before loading the gel (10 μg/sample).

S1 nuclease. This was purchased from Miles Laboratories.

Enzymes sedimentation markers. Beef liver catalase and yeast 3-phosphoglycerate kinase were purchased from Boehringer, and *E. coli* alkaline phosphatase from Worthington Biochemicals. Human blood amylase was obtained from a patient with acute pancreatitis.

DNA cloning and sequencing

*Eco*RI-E fragment of H2 *ts* 125 DNA was cloned in pBR328 and *Hind*III-F fragment in pBR322. Clones were selected according to their susceptibility to various antibiotics and characterized by gel electrophoresis. Nucleotide sequences were derived using the Maxam and Gilbert method. Following the

previously published strategy, both strands have been sequenced (Hérissé *et al.*, 1981). The *Eco*RI-E fragment was hydrolyzed with *Alu*I or *Hha*I. The *Hind*III-F fragment was hydrolyzed by *Hinf*I. Resulting fragments were 5'-labeled using polynucleotide kinase with [γ-^{32}P]ATP.

S1-nuclease analysis of H2 ts 125 mRNA

The S1 analysis was performed according to a modification of the Berk-Sharp S1 mapping procedure, developed by Weaver and Weissmann (1979). Comparative S1 analysis was performed on H2 *ts* 125 and WT whole cytoplasmic mRNA, selected on oligo(dT)-cellulose. The polyadenylated RNA samples were hybridized to the r-strand of the following fragments. *Eco*RI-E recut by *Hha*I (6th fragment, as decreasing mol. wt.); *Hind*III-F/*Hinf*I (4th fragment); these two fragments corresponded to each one of the point mutations. *Eco*RI-E/*Bgl*II, largest fragment, recut by *Sph*I, corresponding to the left-hand moiety of the fiber message; *Hind*III/*Sma*I, corresponding to the right-hand moiety of the fiber mRNA. The fragments were labeled at their 5' end by kinasing, heat-denatured and r-strand isolated on polyacrylamide gel. The r-strand of each fragment was hybridized with either WT or H2 *ts* 125 polyadenylated mRNA, treated with S1 nuclease and analyzed in urea-denaturing polyacrylamide gel.

Gel electrophoresis

Restriction fragments obtained from single and double digestions were analyzed in a 1.3% agarose or 5% polyacrylmide gel as previously described (D'Halluin *et al.*, 1982).

Radioisotopes

L-[^{35}S]Methionine (700–1000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, UK, and [^{3}H]glucosamine (12 Ci/mmol) from the Commissariat à l'Energie Atomique (CEA), Saclay, France. Uniformly ^{14}C-labeled sodium acetate (90–100 mCi/mmol) and uniformly ^{14}C-labeled amino acid mixture (40–50 mCi/matom of carbon) were also purchased from the CEA. [γ-^{32}P]ATP (2900 Ci/mmol) was from NEN, Boston, MA.

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