STUDIES ON PROTEINS INDUCED BY CHICK EMBRYO LETHAL ORPHAN (CELO) VIRUS INFECTION

PART I: Chick Embryo Lethal Orphan (CELO) Virus-induced Early and Late Polypeptides

PART II: Preliminary Characterization of Temperature-sensitive mutants of CELO virus Defective in Viral Protein Production and Transportation.

DOCTRAL THESIS

HIROSHI YASUE

CONTENTS

PART I: Chick Embryo Lethal Orphan (CELO) Virus-induced Early and Late Polypeptides

ABSTRACT

Polypeptides of chick embryo lethal orphan virus (an avian adenovirus) were analyzed with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Virions (ρ=1.338 g/cm^3) and virion-like particles having lower density (light particles, ρ=1.289 g/cm^3) were composed of at least 11 and 14 types of polypeptides, respectively. At least 23 virus-induced polypeptides in infected cells (primary monolayer culture of chicken kidney cells) were detected; eight polypeptides (early polypeptides) were synthesized even in the presence of 1-β-D-arabinofuranosyl cytosine, but the others (late polypeptides) were not. On the basis of migration in SDS-PAGE, five polypeptides were found to be common to virions and infected cells; 5, to light particles and infected cells; 10, to virions and light particles; and 4, to all three. Scarely any of the early polypeptides were not found in the soluble fraction when extracted with the low salt buffer. Some of the early polypeptides were solubilized with the high salt buffer. Other early polypeptides were solubilized by sodium deoxycholate; three of these were found in the so-called 'M-band', as are early polypeptides of human adenoviruses. An experiment with radioactive glucosamine indicated that one of early poypeptides found in the M-band was a glycoprotein.

INTRODUCTION

Chick embryo lethal orphan (CELO) virus, virological properties of which are basically similar to those of human adenoviruses, is classified as an avian adenovirus (reviewed by Slifkin and Merkow, 1973). Several biologically interesting findings concerning this virus have been reported, such as tumorigenicity in hamsters (Sarma et al., 1965), in vitro transformation of human (Anderson et al., 1969a) and hamster (Anderson et al., 1969b) cells, production of tumor-related antigens (Potter and Oxford, 1969; Shild et al., 1970), and potentiation of adeno-associate virus (Ishibashi and Ito, 1971). Furthermore, DNA of this virus has been well studied: 1) it is a linear double-stranded molecule with a molecular weight of 28.3×10^6 (Robinson et al., 1973); 2) it has an inverted terminal repetition at both ends (Robinson and Bellett, 1975); 3) some portions of it have been found to be integrated into the DNA of virus-induced transformed cells (Bellett, 1975; May et al., 1975). However, there is little information available concerning the polypeptides of this virus except for the findings of Laver et al. (1971). These investigators found that the virions purified from allantoic fluid had at least five kinds of polypeptides, the largest of which was identified as hexon polypeptide by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The migration of virion polypeptides in SDS-PAGE showed considerable differences from those of adenovirus type 2.

As one of us reported (Ishibashi, 1970, 1971), we isolated 49 temperature-sensitive (ts) mutants of this virus, and preliminarily classified them into five groups on the basis of properties such as

viral antigen productivity and transportability of viral antigen from cytoplasm to nucleus. In order to utilize these mutants for elucidating the regulatory mechanism of viral infection, it becomes necessary to have available a general catalogue of the polypeptides of this virus. Therefore we used an SDS-PAGE technique to examine the polypeptide composition not only of virions but also of virion-like particles having lower density (light particles) and the virus-induced early and late polypeptides in infected chicken kidney cells.

MATERIALS AND METHODS

Cell culture

Primary monolayer cultures of chicken kidney cells (CKC) were prepared as described previously (Ishibashi, 1971) except that Eagle's minimum essential medium (MEM) (Eagle, 1959) containing 10 mM N-2-hydroxylpiperadine-N'-2-ethane sulfonic acid (HEPES), pH 7.2, and 5% bovine serum was used for cell growth.

Virus

The wild type CELO virus described in the previous paper (Ishibashi, 1971) was used for the present experiments. Its infectivity was titrated by the plaque method as described previously (Ishibashi, 1971).

Preparation of radioactive virions and light particles for SDS-PAGE analysis

CKC (a total of 4.6×10^7 cells in 20 petri-dishes, each having a diameter of 90 mm) were adsorbed with CELO virus at 37° for 2 hr at

a multiplicity of infection of 20 plaque-forming units (PFU) in the serum-free MEM (1.6 ml in each petri-dish). After the adsorption, infected CKC were incubated at 37° in MEM containing 2% bovine serum until the labeling experiment was begun. Twenty-two hours after infection the culture medium of each petri-dish was replaced with 5 ml of leucine-free MEM plus 2.5 μCi [^{14}C]leucine (Daiichi Pure Chemicals, 280 mCi/mmole) and 2% dialyzed bovine serum, and the incubation was continued for another 5 hr. Cultures were then treated with non-radioactive leucine so as to attain one-tenth the leucine concentration normally present in MEM and incubated for an additional 22 hr. After the labeling infected CKC were scraped from the glass surface of the petri-dishes and collected by low-speed centrifugation (250 x g, 10 min, 4°). The collected cells were suspended in 6 ml of 10 mM Tris-HCl (pH 8.0 at 25°) and then treated with 6 ml of chloroform. The mixture was vigorously agitated and subjected to centrifugation (1,500 x g, 15 min, 4°) to separate the aqueous and chloroform phases. The aqueous phase was then layered on top of a preformed linear CsCl gradient ranging in density from 1.225 to 1.425 g/cm^3, and centrifuged for 3 hr at 23,000 rpm in a Beckman SW25.1 rotor. Previous studies had shown that these conditions could be used for the purification of adenovirus type 2 virions with virtually no contamination by host cellular materials (Maizel, 1968; Ishibashi and Maizel, 1974a). The virus band (1.338 g/cm^3) and light particle band (1.289 g/cm^3) were collected by piercing the bottom of the centrifuge tube. Virions and light particles were re-banded in another preformed linear CsCl gradient ranging in density from 1.225 to 1.400 g/cm^3 (Beckman SW41 rotor,

35,000 rpm, 2 hr, 4°). The purified virions and light particles were dialyzed against 10 mM Tris-HCl (pH 8.0 at 25°) at 4° and then re-dialyzed against the SDS-sample buffer described in the SDS-PAGE technique section.

Labeling of infected or mock-infected CKC for virus-induced polypeptide analysis

CKC were adsorbed with CELO virus as described in the preceding section. The multiplicity of infection was as described in the legends to figures. After the adsorption, infected or mock-infected CKC were incubated at 40° in MEM containing one-tenth the normal concentration of methionine or leucine, 2% dialyzed bovine serum, and either 0 or 20 μg/ml 1-β-D-arabinofuranosyl cytosine (Ara C). Cultures were washed twice with phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) and then incubated in the radioactive medium containing appropriate amounts of [^{14}C]leucine (280 mCi/mmole) or [^{35}S]methionine (381 Ci/mmole, New England Nuclear) for appropriate periods at 40° in the presence or absence of Ara C (20 μg/ml). (Precise details of the experimental procedures are described in the legends to figures.) After the labeling, cells were collected as described in the preceding section.

Fractionation of proteins from whole cells with salt and detergents (SD-fractionation)

CKC were scraped off the surfaces of petri-dishes and washed once with PBS by low-speed centrifugation. Pelleted cells were suspended in buffer L (10 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.6 at 25°) and sonicated for 1 min in an ice bath to

disrupt cells. The resulting sample was subjected to centrifugation in a Beckman SW50.1 rotor at 30,000 rpm for 60 min at 4° to separate the supernatant from the pellet. The supernatant fraction was removed and designated 'low salt fraction'. The pellet was suspended in buffer L containing 1.7 M NaCl and sonicated for 1 min in an ice bath to disperse the pellet; this was followed by centrifugation under the same conditions as described above. The supernatant fraction was removed and designated 'high salt fraction'. The pellet was suspended in buffer L containing 50 mM NaCl and 1% Triton X-100, and again subjected to sonication and centifugation under the same conditions. This supernatant fraction was removed and designated 'Triton X-100 fraction'; the pellet was treated with buffer B [50 mM NaCl, 5 mM EDTA, 0.4% sodium deoxycholate (DOC), 20 mM Tris-HCl, pH 8.0 at 25°], sonicated, and centrifuged under the same conditions. The supernatant fraction was removed and designated 'DOC fraction'. The resulting pellet was designated 'precipitated fraction'. These fractions were used for SDS-PAGE analysis and Sephadex G-200 column chromatography.

Separation of 'nuclei' from 'cytoplasm' by Brij-58 treatment

CKC were scraped from the surfaces of petri-dishes and washed once with PBS by low-speed centrifugation. Pelleted cells were suspended in reticulocyte standard buffer (RSB, 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2 at 25°) by gentle agitation so that the final concentration of cells was 2.7×10^5 per ml; they were allowed to stand for 15 min in an ice bath. To the cell suspension was added Brij-58 (final concentration 1%). The mixture was agitated with a Vortex mixer

for 5 sec and allowed to stand for 5 min at room temprerature. Samples were centrifuged to sediment 'nuclei' (250 x g, 5 min, 4°). The supernatant was collected and designated 'cytoplasm'.

Separation of 'nuclei' from 'cytoplasm' with Nonidet P-40 (NP-40) treatment

The method used was that described by Shiroki et al. (1974).

SDS-PAGE technique

SDS-PAGE was conducted according to the method described by Maizel (1971). The SDS-disc system was employed with a gel concentration of 12%; the ratio of acrylamide to bis-acrylamide was 30 to 0.8. Samples to be analyzed were concentrated by the cold 10% trichloroacetic acid (TCA) precipitation procedure as described by Maizel (1971). Electrophoresis was conducted at 100 V and was continued until the phenol red marker reached the anodal end of the gel.

RESULTS

Polypeptides of virions and light particles

The monolayer cultures of CKC infected with CELO virus at 40° were labeled with [^{14}C]leucine between 22 and 49 hr after infection. Virions and light particles were purified by two cycles of CsCl density gradient centrifugation by the procedures described in Materials and Methods. Virions and light particles were discretely banded at densities of 1.338 g/cm^3 and 1.289 g/cm^3, respectively, as reported by Anderson et al. (1971). Purified virions and light particles were subjected to SDS-PAGE to determine the polypeptide composition. Figure 1 shows the autoradiogram of the gel. Though some of weak bands may not be seen well in the figure, at least 11 bands in virions and 14 bands in light particles were detected in the original X-ray film. Individual bands observed in the autoradiogram corresponded to Coomassie Brilliant Blue-stained bands (not shown in figures), and the relative intensities of individual bands in the autoradiogram were similar to those in the stained gel. There were 10 common polypeptide bands between virions and light particles, as shown in Fig. 1 and Table 1. Of these, only band V115K, the major virion polypeptide, had been previously identified; it consists of hexon polypeptide (Laver et al., 1971). The location of other polypeptides in virions and light particles has not been clarified.

Time course of virus-induced polypeptide analysis

CKC were infected with CELO virus or were mock-infected at 40° and labeled with [^{35}S]methionine or [^{14}C]leucine for 1 or 12 hr at various times after infection (see legend to Fig. 2). The temperature

employed in this experiment was the same as the restrictive temperature for ts-mutants we had isolated (Ishibashi, 1971). The growth of the wild type virus used in this experiment at 40° was not less than that at 37° (our unpublished data). Polypeptides in labeled cells were analyzed by means of SDS-PAGE. Figure 2 shows autoradiographic results of one of such experiments with [^{35}S]methionine labeling. In this type of analysis virus-induced polypeptide bands often overlaped bands which could be observed even in mock-infected cells. In such case it was impossible to determine whether polypeptides which were synthesized even in mock-infected cells were induced to synthesize more due to virus infection, or polypeptides which were not synthesized in mock-infected cells were induced to synthesize. Whichever was the case, bands whose intensity began to increase at various times after virus inoculation were operationally defined as virus-induced polypeptide bands. Based on this criterion, at least 14 virus-induced bands were detected in Fig. 2. Bands E84K and E38K were detected in infected cells pulse-labeled for 1 hr from 6 hr to at least 28 hr post infection. Bands L150K, L115K, and L54.5K became detectable between 14 and 15 hr after infection. Other virus-induced bands became detectable between 18 and 19 hr after infection or later. All these bands except L48K were detected also in infected cells pulse-labeled from 18 to 30 hr after infection. The migration of band L115K (Fig. 1) corresponded to that of V115K (hexon polypeptide). A Coomassie Brilliant Blue-stained band corresponding to E84K became detectable approximately 14 hr after infection; stained bands corresponding to L150K, L115K, and L30K, about 21 hr after infection; and a stained band corresponding

to L17.5K, about 30 hr after infection. These stained bands (not shown) became more intense with the passage of time after infection. Other virus-induced bands did not accumulate sufficiently to be detectable in the stained gel. Since polypeptides containing no methionine would be missed in the ^{35}S-labeling experiment, infected or mock-infected cells were pulse-labeled with [^{14}C]leucine between 20 and 21 hr after infection in a parallel experiment. Polypeptides with ^{14}C-label were analyzed by means of SDS-PAGE. As shown in Fig. 3 bands L76K, L48K, L30K, and L29K, which had been very weakly labeled, if at all, with [^{35}S]methionine (Fig. 1 and Fig. 2), were more strongly labeled with [^{14}C]leucine. Bands L94K, L27K, and L23K were detected only in infected cells labeled with [^{14}C]leucine. In an experiment in which infected or mock-infected cells were pulse-labeled with [^{14}C]leucine for 2 hr at various times after infection, these 7 bands (L94K, L76K, L48K, L30K, L29K, L27K, and L23K) became detectable between 16 and 18 hr post infection or earlier (autoradiograms are not shown). In experiments with [^{14}C]leucine labeling including the one shown in Fig. 3, the band L14K was not unequivocally demonstrated. All these results are summarised in Table 1.

Virus-induced polypeptides formed in the presence of an inhibitor of DNA synthesis

1) Analysis at the whole cell level

CKC in a medium containing Ara C were infected with CELO virus or were mock-infected at 40° and labeled with [^{35}S]methionine or [^{14}C]leucine for 1 or 22 hr at various times after infection, as

indicated in the legend to Fig. 4. Polypeptides in labeled cells (whole cell sample) were compared by means of SDS-PAGE. A portion of the results is shown in Fig. 4. Bands E84K and E38K, which had been detected in 1-hr pulse-labeled cells beginning 6 hr after infection (Fig. 2), could also be detected in this experiment. They were first apparent in the sample pulse-labeled with [^{35}S]methionine between 4 and 5 hr after infection, and they continued to be detected in 1-hr pulse-labeled samples until at least 21 hr after infection. Moreover, they were detected in the long-pulse-labeled sample from 2 to 24 after infection. In addition to E84K and E38K, ^{35}S-labeled bands designated E55K, E54K, E45K, and E15.5K could be detected in whole cell samples obtained during similar experiments (e.g., Figs. 5 and 9). These components, however, were not detected in the experiment shown in Fig. 4. In fact, the infection-specific bands designated with the heading "L" in Fig. 1 were not produced in any detectable quantity in this type of experiment. [The polypeptides, whose synthesises were not inhibited even in the presence of Ara C, were designated 'early polypeptides' as in the case of human adenovirus (Walter and Maizel, 1974); and the others, 'late polypeptides'.] In the stained gel from which the autoradiogram shown in Fig. 4 was produced, a band corresponding to E84K became visible approximately 11 hr after infection and became intense as a function of time after infection (stained electropherogram not presented). A similar experiment, in which [^{14}C]leucine was used as the label, revealed no additional infection-specific bands.

2) Fractionation of proteins from whole cells with salt and detergents (SD-fractionation)

In order to study the early polypeptides (see above) more precisely and to detect other early polypeptides, infected and mock-infected cells which had been labeled with [^{35}S]methionine at 40° from 2 to 24 hr after infection in the presence of Ara C were fractionated as described in Materials and Methods. Fractionated samples contain fewer protein species than non-fractionated samples (or whole cell samples), and, therefore, minor proteins can be detected more readily in the former when the samples are analyzed by SDS-PAGE. Results of one of such experiments are shown in Fig. 5 and summerized in Table 2. The percentage of the TCA-insoluble radioactivity found in each fraction is also given in Table 2. Bands E55K and E54K were detected mainly in the high salt fraction. (The preferential recovery of these bands in the high salt fraction were more clearly demonstrated in an independent experiment not shown here.) Band E45K was detected in the high salt fraction, in the DOC fraction, possibly in the precipitated fraction. Band E84K was detected in the high salt fraction, but the intensity was very low (Fig. 5); this band was much more prominent in the DOC fraction and in the precipetated fraction. Other early bands (E38K, E15.5K, E10.5K, and E9.5K) were detected in the DOC fraction and the precipitated fraction. On the basis of migration in SDS-PAGE, none of bands (E84K, E55K, E54K, E45K, E38K, E15.5K, E10.5K, and E9.5K) corresponded to those of virions or of light particles (Table 1).

3) Fractionation of cells into 'cytoplasm' and 'nuclei'

CKC were infected with CELO virus or were mock-infected and labeled as in the preceding section (labeling times indicated in legend to Fig. 6). They were then fractionated into 'nuclei' and 'cytoplasm' by treatment with detergents, as described in Materials and Methods, and these

fractions were examined by SDS-PAGE. Results obtained with Brij-58 and NP-40 are shown in Figs. 6 and 8, respectively; major findings are summarized in Table 3. Briefly, E84K was detected more or less in both 'cytoplasm' and 'nuclei' under all conditions studied, as was E38K in the longer labeling experiments. However, E55K and E54K showed differences in the distribution of labeling depending on the treatments of detergents employed, indicating that the fractionation was not absolute. In addition to the above findings, a band designated X_1 and a weak band, which probably corresponded to either E10.5K or E9.5K, were detected in the nuclear fraction obtained with Brij-58 treatment of infected cells labeled between 5 and 24 hr after infection (Fig. 6). Band X_1 was not reproducibly observed in infected samples and apparently did not correspond to any of the early bands mentioned above. Other early polypeptides (E45K, E10.5K, and E9.5K) were not detected in these fractionation procedures.

4) M-band technique

In the case of human adenovirus type 2 and type 12, viral DNA synthesizing activity was recovered in the fraction called 'M-band' (Shiroki et al., 1974; Yamashita and Green, 1974), and some of the early polypeptides induced by adenovirus type 2 were found also in this fraction (Yamashita and Green, 1974). In order to determine whether or not some of the CELO virus-induced early polypeptides were recovered in the M-band fraction, such a fraction was prepared from a portion of the infected and mock-infected cells used in the preceding experiment. The nuclear fraction in which E84K and E38K had been detected (Table 3), i.e., that obtained by the NP-40 treatment, was mixed with sodium sarcocinate and $MgCl_2$, applied to the top of the upper sucrose layer

(15% w/v and 47% w/v, respectively, were employed), and subjected to centrifugation. The sedimentation pattern (Fig. 7) revealed two major peaks of radioactivity: one at the top of the centrifuge tube (fractions 1 and 2, 'Top-fraction'); the other, at the interface between sucrose layers (fraction 7, M-band). The ratio of radioactivity of the M-band to the top-fraction in the infected sample was higher than that in the mock-infected sample, implying that the M-band of the infected sample contained some infection-specific polypeptides. As shown in Fig. 8, when SDS-PAGE was carried out, three polypeptide bands (E84K, E38K, and E15.5K) were detected in the M-band fraction of the infected sample, but no early polypeptide bands were detected in the Top fraction.

Labeling with [^{14}C]glucosamine

CKC in a medium containing Ara C were infected with CELO virus or were mock-infected and labeled with [^{14}C]glucosamine or with [^{35}S]methionine at 40° between 2 and 24 hr after infection (see legend to Fig. 9). The autoradiogram (Fig. 9) obtained after SDS-PAGE showed two infection-specific bands labeled with [^{14}C]glucosamine. One was an intensely labeled band which showed the same migration as E84K; the other was a weakly labeled band which migrated at a rate similar, but not identical, to that of E15.5K. These [^{14}C]glucosamine labeled bands, like E84K and E15.5K, were not solubilized efficiently by buffer L (low salt buffer) or buffer L containing 1.7 M NaCl (high salt buffer). When CKC were infected with CELO virus in the absence of Ara C, labeled with [^{14}C]glucosamine at a late stage of infection (from 18 to 24 hr post infection), and then analyzed with SDS-PAGE, only one infection-specific

^{14}C-band was detected (electropherogram not presented); it corresponded to E84K. These lines of evidence strongly indicate that E84K is a glycoprotein. Further studies are required to clarify the relationship between the weakly labeled band and E15.5K.

DISCUSSION

Laver et al., (1971), using SDS-PAGE in Tris-boric acid-EDTA buffer, identified at least 5 kinds of polypeptides in CELO virions. In this study we have not only re-examined the polypeptide composition of virions but have also determined that of light particles and examined the virus-induced early and late polypeptides in infected CKC. The technique employed in the present work involved the high-resolution SDS-PAGE system in Tris-glycine buffer previously described by Maizel (1971). As summarized in Table 1, we identified at least 11 polypeptides in virions, 14 polypeptides in light particles, and 23 virus induced-polypeptides in infected cells. Experiments with Ara C indicated that 8 of the virus-induced polypeptides were early, whereas the others were late. When virion polypeptides, light particle polypeptides, and the virus-induced polypeptides in infected cells were compared on the basis of their migration rates in SDS-PAGE, it was found that virions and infected cells had 5 polypeptides in common; light particles and infected cells, 5; virions and light particles, 10; and that 4 polypeptides were common to all these sources. These relationships resembled those observed in human adenovirus type 2 infection (Ishibashi and Maizel, 1974a), implying that light particles were a precursor of virions. Since the virion polypeptides examined in the present study exhibited considerable differences in migration in SDS-PAGE, compared with those of human adenovirus type 2, we could not deduce localizations of polypeptides (except hexon polypeptide) in CELO virions by analogy with the human adenovirus.

If we make the following assumptions: 1) ratio of the number of [^{14}C]leucine residues to that of other amino acid residues in the

polypeptides comprising virions were constant (an assumption supported by the observation that the distribution of radioactivity was similar to that of Coomassie Brilliant Blue staining); 2) virions were constructed of 240 hexon units (Valentine and Periera, 1965); and 3) a hexon unit was composed of 3 hexon polypeptides (Maizel et al., 1968), we can use the densitometric patterns of autoradiograms produced by contacting the gel to X-ray film for various periods of time to calculate the number of molecules of each polypeptide present in one virion. Results of these calculations (Table 4) were compared with those made for human adenovirus type 2 (Table 4). However, although the values obtained span essentially the same range in both cases, it is still necessary to determine the locations of the individual polypeptides in virions.

In human adenovirus type 2 and 5 infection, no virus-induced band migrating slower than hexon polypeptide was detected in SDS-PAGE (Anderson et al., 1973; Walter and Maizel, 1974; Russel and Skehel, 1972), but in the case of CELO virus infection, such a band was observed. The migration rate of this band did not change even after infected samples were incubated at 100° in the SDS-sample buffer for 45 sec longer than the routine period of 60 sec, suggesting that L150K was not a 'meta-stable protein complex' (Maizel, 1971) but a single polypeptide chain.

If it is postulated that all 23 virus-induced polypeptides detected in the present study are coded by the CELO virus genome and that there is no precursor-product relationship among these polypeptides, the combined molecular weight of the early polypeptides (3.1×10^5) should correspond to about 23% of the asymmetric coding capacity of the viral genome.

In the [^{14}C]glucosamine experiment, early polypeptide E84K became labeled, but the late polypeptides did not. This was different from the case of human adenovirus type 2 infection in that a late polypeptide (fiber polypeptide), as well as one of the early polypeptides (E2) whose molecular weight was 19K, had been found to be labeled with radioactive glucosamine (Ishibashi and Maizel, 1974b).

When proteins of infected cells incubated in the presence of Ara C were extracted successively with low salt, high salt, Triton X-100, and DOC (Table 2), Scarcely any of the early polypeptides were extracted by the low salt, indicating that they were associated with subcellular organelles. E55K and E54K were solubilized by high salt; the major portion of E45K was solubilized by high salt and DOC, and the rest of this polypeptide remained in the precipitated fraction. These results indicated that polypeptides E55K and E54K are bound electrostatically to subcellular organelles. (These organelles must have been recovered in the nuclear fraction obtained by the Brij-58 treatment but in the cytoplasmic fraction obtained by the NP-40 treatment--see Table 3.) By contrast, E45K is apparently bound to subcellulat structures in some different way, possibly involving hydrophobic interaction. Other virus-induced early polypeptides (E84K, E38K, E15.5K, E10.5K, E9.5K) were not solubilized with low salt, high salt, or Triton X-100; however more than 50% of each was solubilized by DOC, which suggested that these polypeptides were embedded in membrane structures. E84K, E38K, and E15.5K polypeptides were detected in the so-called 'M-band' fraction. In the case of human adenovirus type 12 infection, the M-band was suggested to contain not only nuclear membrane but also

other hydrophobic components in which the viral DNA was synthesized (Shiroki et al., 1974). Thus this evidence also suggested that E84K, E38K, and E15.5K were integrated into the nuclear membrane or other hydrophobic components of the nucleus.

Further experimentation will be required to achieve precise characterization of the early CELO virus-induced polypeptides, and to determine whether any of them shows single-stranded DNA-binding ability or not. Such ability was demonstrated in the major early polypeptide (molecular weight 72,000) induced by adenovirus type 2 (van der Vliet and Levine, 1972).

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fractions containing newly synthesized viral DNA and proteins. J. Virol. 14, 412-420.

legend to Fig. 1.

Comparison of polypeptides in virions and light particles, and those induced in virus-infected cells.

Virions and light particles were purified, by 2 cycles of CsCl density gradient centrifugation, from infected CKC which had been labeled with [^{14}C]leucine at 37° from 22 to 49 hr post infection. Procedures are described in detail in Materials and Methods. The gel electrophoresis of polypeptides from infected cells is the same sample as that shown in Fig. 2 for infected cells labeled from 27 to 28 hr post infection. The figure shows the autoradiogram of the gel. In this and all succeeding electropherograms, the bottom of the figure corresponds to the anodal end of the gel. Molecular weights of polypeptides were calculated by comparison with the migration of standard proteins: myosin (MW 215,000), β-galactosidase (MW 135,000), bovine albumin (MW 67,000), ovalbumin (MW 43,500), cytochrome c (MW 12,700), and polypeptides of human adenovirus type 2 virion. The following nomenclature was used throughout this study: virion polypeptides are designated "V"; polypeptides of light particles, "Lp"; virus-induced polypeptides synthesized in the presence of Ara C (20 ug/ml), "E"; virus-induced polypeptides not detected in the presence of Ara C, "L"; the accompanying Arabic numeral represents the molecular weight in thousands.

legend to Fig. 2.

Time course of virus-induced polypeptide synthesis in infected CKC at 40°.

CKC, 5.5 x 10^5 cells per petri-dish (42 mm in diameter), were

infected (I) with the virus or were mock-infected (M) at a multiplicity of 50 PFU. Infected and mock-infected CKC were labeled with [^{35}S]methionine at 40° for 1 or 12 hr at times indicated in the figure (hr post infection). The radioactive medium used in each petri-dish for 1-hr pulse-labeling was 1 ml of methionine-free MEM containing 5 μCi [^{35}S]methionine; that used for 12-hr pulse-labeling was 2 ml of methionine-free MEM supplemented with one-tenth the usual amount of methionine and 5 μCi [^{35}S]methionine. Samples were processed as described in Materials and Methods and analyzed by SDS-PAGE. The figure shows the autoradiogram of the gel.

legend to Fig. 3.

Autoradiographic comparison of virus-induced polypeptides labeled with [^{14}C]leucine or [^{35}S]methionine.

Infected (I) and mock-infected (M) cells were pulse-labeled with either [^{14}C]leucine or [^{35}S]methionine for 1 hr at 20 hr post infection. Procedures of infection and labeling were the same as in Fig. 2.

legend to Fig. 4.

Time course of the virus-induced early polypeptide synthesis in infected CKC incubated in the presence of Ara C (20 μg/ml).

CKC, 5.5 x 10^5 cells per petri-dish, were infected (I) with the virus or were mock-infected (M) at a multiplicity of 100 PFU in the presence of Ara C (20 μg/ml). Infected and mock-infected CKC were labeled with [^{35}S]methionine for 1 or 22 hr at times indicated in the figure (hr post infection). The radioactive medium used in each petri-dish for 1-hr pulse-labeling was 1 ml of methionine-free MEM containing

5 μCi [^{35}S]methionine and Ara C (20 μg/ml). For 22-hr pulse-labeling, the radioactive medium used in each petri-dish was 2 ml of methionine-free MEM supplemented with one-tenth the usual amount of methionine, 5 μCi [^{35}S]methionine, 20 μg/ml Ara C, and 5% dialyzed bovine serum. The figure shows the autoradiogram of the gel obtained by SDS-PAGE. Only a portion of the results of this experiment is presented in the figure.

legend to Fig. 5.

Distribution of virus-induced early polypeptides in fractions of infected CKC incubated in the presence of Ara C (20 μg/ml).

CKC (6.9 x 10^6 cells in 3 petri-dishes, each having a diameter of 90 mm) were infected (I) with the virus or were mock-infected (M) at a multiplicity of 100 PFU in the presence of Ara C (20 μg/ml) and then labeled with [^{35}S]methionine at 40° from 2 to 24 hr post infection. The radioactive medium used in each petri-dish was 5 ml of methionine-free MEM supplemented with one-tenth the usual amount of methionine, 20 μCi [^{35}S]methionine, 20 μg/ml Ara C, and 5% dialyzed bovine serum. Proteins in infected and mock-infected CKC were extracted successively with low salt, high salt, Triton X-100, and DOC. Procedures are described in detail in Materials and Methods. The resulting samples were analyzed with the SDS-PAGE. The figure shows the autoradiogram of the gel. low salt, low salt fraction; high salt, high salt fraction; Triton X-100, Triton X-100 fraction; DOC, DOC fraction; precipitate, precipitated fraction.

legend to Fig. 6.

Localization of virus-induced early polypeptides in 'cytoplasm' and 'nuclei' by treatment with Brij-58.

CKC (5.5×10^5 cells/petri-dish) were infected (I) with the virus or were mock-infected (M) at a multiplicity of 100 PFU in the presence of Ara C and then labeled with [^{35}S]methionine for 1 or 19 hr at various times post infection. For 1-hr pulse-labeling each petri-dish of cells contained 1 ml of methionine-free MEM supplemented with 5 μCi [^{35}S]methionine and Ara C (20 μg/ml). For 19-hr pulse-labeling, the radioactive medium was 2 ml of methionine-free MEM supplemented with one-tenth the usual amount of methionine, 5 μCi [^{35}S]methionine, 20 μg/ml Ara C, and 5% dialyzed bovine serum. The harvested cells were treated with 1% Brij-58 to prepare 'nuclei' (N) and 'cytoplasm' (C) as described in Materials and Methods. The figure shows the autoradiogram of the gel obtained by SDS-PAGE. X_1 represents an unidentified polypeptide band observed in the autoradiogram.

legend to Fig. 7.

Preparation of 'M-band' from infected and mock-infected CKC incubated in the presence of Ara C.

CKC (2.3×10^6 cells in a petri-dish with a diameter of 90 mm) were infected with the virus or were mock-infected at a multiplicity of 100 PFU in the presence of Ara C (20 μg/ml) and incubated from 2 to 24 hr post infection in 5 ml of methionine-free MEM supplemented with one-tenth the usual amount of methionine, 10 μCi [^{35}S]methionine, 20 μg/ml of Ara C, and 5% dialyzed bovine serum. Nuclei were isolated

by the NP-40 treatment and fractionated by means of the M-band technique (Shiroki et al., 1974). After centrifugation, fractions of 0.8 ml each were collected from the top of the centrifuge tube, and the TCA-insoluble radioactivity in the fractions was setermined. Solid line, infected sample; broken line, mock-infected sample.

legend to Fig. 8.

Autoradiogram of fractions obtained after NP-40 treatment and after M-band technique.

The 'M-band' (fraction 7) and 'Top-fraction' (fraction 1 and 2) shown in Fig. 7, as well as the 'cytoplasm' and 'nuclei' were analyzed by means of SDS-PAGE. Cyt., 'cytoplasm prepared by the NP-40 treatment; Nuc., 'nuclei' prepared by the NP-40 treatment; I, infected sample; M, mock-infected sample.

legend to Fig. 9.

[^{14}C]glucosamine labeling of infected and mock-infected CKC in the presence of Ara C (20 μg/ml). CKC (2.3 x 10^6 cells in a petri-dish having a diameter of 90 mm) were infected (I) with the virus or were mock-infected (M) at a multiplicity of 100 PFU in the presence of Ara C (20 μg/ml) and then labeled with 5 μCi [^{35}S]methionine (381 Ci/mmole) or 15 μCi [^{14}C]glucosamine (56.5 mCi/mmole, Daiishi Pure Chemicals) from 2 to 24 hr post infection at 40°. The radioactive medium for [^{14}C]glucosamine labeling was 5 ml of MEM containing 15 μCi [^{14}C]glucosamine

labeling was 5 ml of MEM containing 15 μCi [^{14}C]glucosamine and 5% dialyzed bovine serum; that for [^{35}S]methionine labeling was 5 ml of methionine-free MEM supplemented with one-tenth the usual amount of methionine, 5 μCi [^{35}S]methionine, and 5% dialyzed bovine serum.

title to Table 1.

Polypeptides of virions and light particles, and virus-induced polypeptides.

title to Tabel 2.

Extraction of virus-induced early polypeptides with salt and detergents.

title to Table 3.

Localization of virus-induced early polypeptides in infected cells.

title to Table 4.

Estimated molecular content of polypeptides in the virion

legend to Table 4.

1) Vualues calculated from densitometric patterns of autoradiograms which were produced by contacting the gel to X-ray film for various periods of time. The following assumptions were made; a) ratio of the number of residues to other amino acid residues in polypeptides comprising the virion were constant; b) the virion was constructed of 240 hexon units; and c) a hexon unit was composed of 3 hexon polypeptides.

2) Values reported by Maizel *et al*. (1968).

3) Values reported by Everitt *et al*. (1973).

Table 1

Early polypeptide		Virus-induced polypeptide Late polypeptide		Light particles	Virions
[^{35}S]Met	[^{14}C]Leu	[^{35}S]Met	[^{14}C]Leu	[^{14}C]Leu	[^{14}C]Leu
		L150K	L150K		
		L115K	L115K	Lp115K	V115K
		(-)	L94K	Lp94K	V94K
E84K	E84K				
		L76K	L76K	Lp76K	V76K
				Lp72K	V72K
				Lp66K	V66K
				Lp64K	V64K
				Lp57K	V57K
E55K	ND				
		L54.5K	L54.5K		
E54K	ND				
		L51K	L51K	Lp51K	V51K
		L48K	L48K		
E45K	ND				
				Lp42K	
E38K	E38K				
				Lp37K	
		L32K	L32K	Lp32K	
		L30K	L30K		
		L29K	L29K		
		(-)	L27K		
				Lp24K	
		(-)	L23K		
		L17.5K	L17.5K		
				Lp16.5K	V16.5K
		L16K	L16K		
E15.5K	ND				
		L14K	?		V14K
				Lp12K	V12K
E10.5K	ND				
E9.5K	ND				

(-), not detected; ND, cell-fractionation to detect corresponding polypeptide was not done.

Table 2

Poly-Peptide	Whole cell 100%	Fraction: Low salt 16.4%	High salt 26.7%	Triton 33.8%	DOC 9.2%	Precipitate 13.8%
E84K	±	-	±	-	+	+
E55K	+	±	+	-	-	-
E54K	+	-	+	-	-	-
E45K	+	-	+	-	+	±
E38K	+	-	-	-	+	+
E15.5K	-	-	-	-	+	+
E10.5K	-	-	-	-	+	+
E9.5K	-	-	-	-	+	+

+, clearly detected; ±, detected but not clearly; -, not detected.

Percentage values indicate distribution of TCA-insoluble radioactivity.

Table 3

Treatment	Brij-58		Brij-58		NP-40	
Labeling time	5-6 hr		5-24 hr		2-24 hr	
Fraction	N	C	N	C	N	C
E84K	+	+	++	+	++	±
E55K	-	-	+	-	-	+
E54K	-	-	+	-	-	±
E45K	-	-	-	-	-	-
E38K	+	±	+	+	++	+
E15.5K	-	-	±	±	-	-
E10.5K	-	-	] +	] -	-	-
E9.5K	-	-			-	-

++, clearly detected as heavy band

+, Clearly detected

±, detected but not clearly

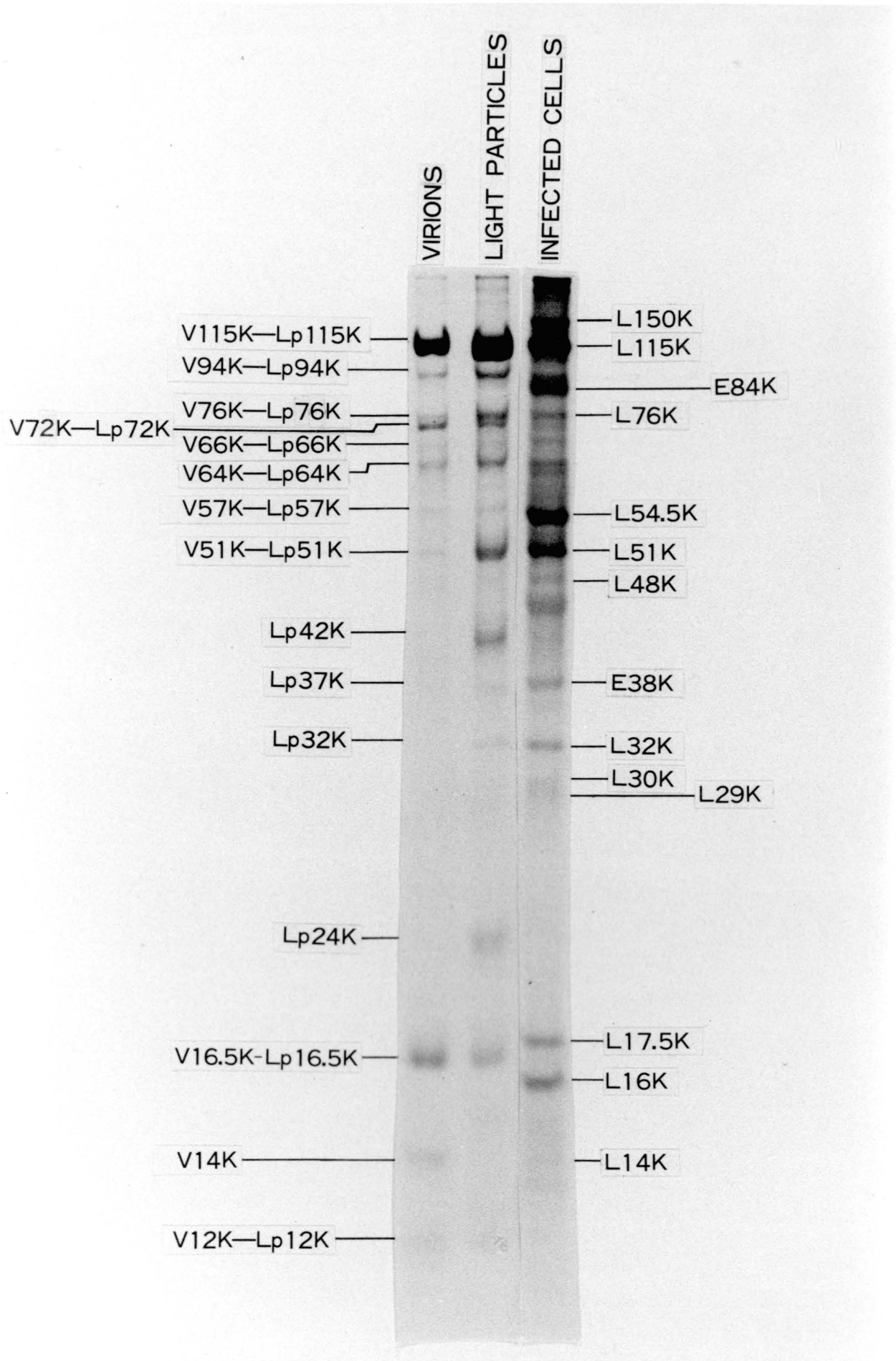
-, not detected

C, cytoplasm; N, nucleus.

Table 4

CELO virus		Human adenovirus type 2	
Poly-peptides	Molecules[1] Virion	Poly-peptides	Molecules Virion
V115K	720	II (MW 120000)	720[2]
V94K	64	III (MW 70000)	96[2]
V76K		IV (MW 62000)	96[2]
V72K	120	V (MW 48500)	180[3]
V66K	42	VI (MW 24000)	420[3]
V64K	110	VII (MW 18500)	1070[3]
V57K	40	X (MW 6500)	50[3]
V51K	63		
V16.5K	1010		
V14K	560		
V12K	610		

Fig 1


VIRIONS
LIGHT PARTICLES
INFECTED CELLS
V115K—Lp115K
V94K—Lp94K
V76K—Lp76K
V72K—Lp72K
V66K—Lp66K
V64K—Lp64K
V57K—Lp57K
V51K—Lp51K
Lp42K
Lp37K
Lp32K
Lp24K
V16.5K-Lp16.5K
V14K
V12K—Lp12K
L150K
L115K
E84K
L76K
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L30K
L29K
L17.5K
L16K
L14K

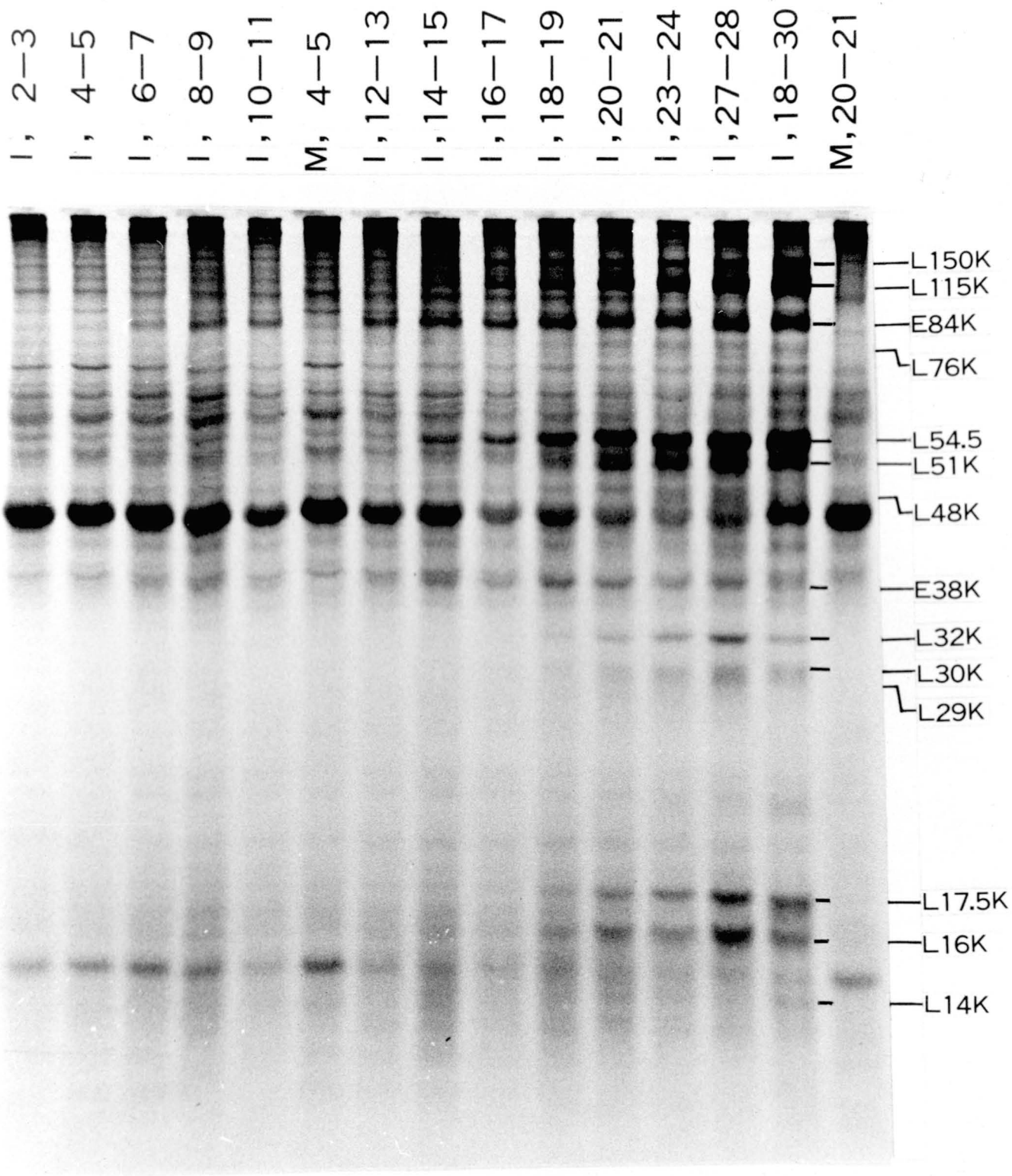
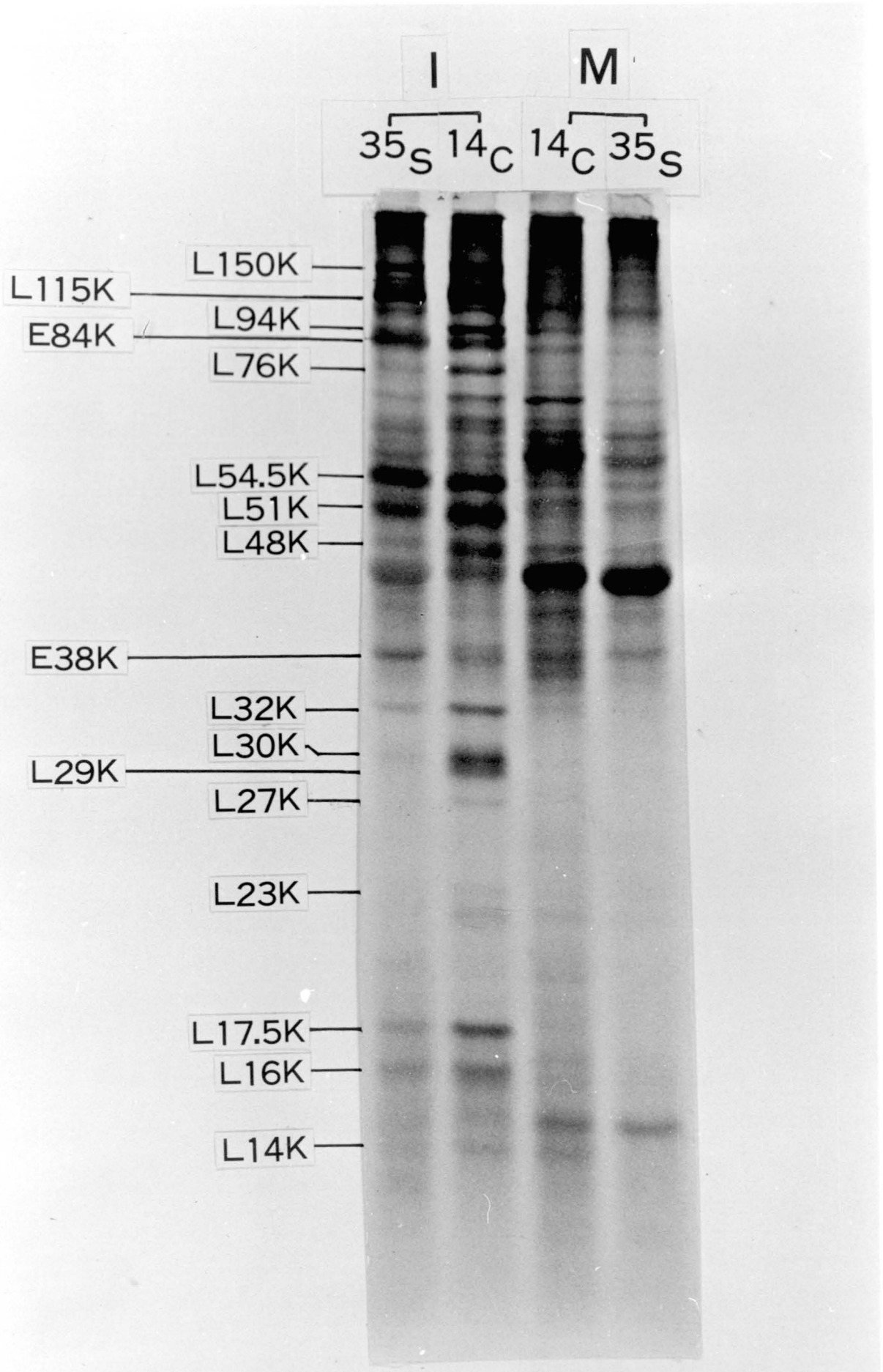

I, 2—3
I, 4—5
I, 6—7
I, 8—9
I,10—11
M, 4—5
I,12—13
I,14—15
I,16—17
I,18—19
I,20—21
I,23—24
I,27—28
I,18—30
M,20—21
L150K
L115K
E84K
L76K
L54.5
L51K
L48K
E38K
L32K
L30K
L29K
L17.5K
L16K
L14K

Fig 3


I
M
35S 14C
14C 35S
L150K
L115K
L94K
E84K
L76K
L54.5K
L51K
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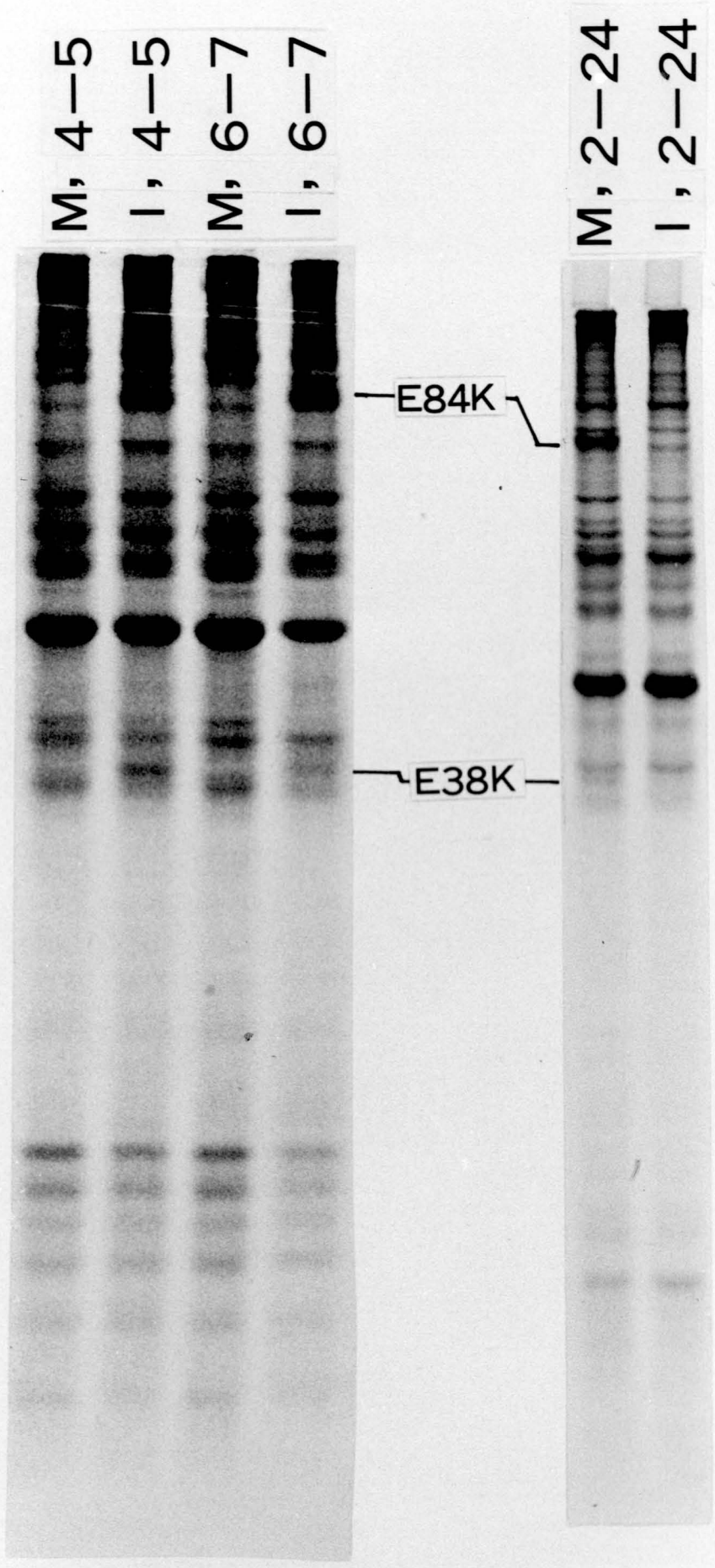
L29K
L27K
L23K
L17.5K
L16K
L14K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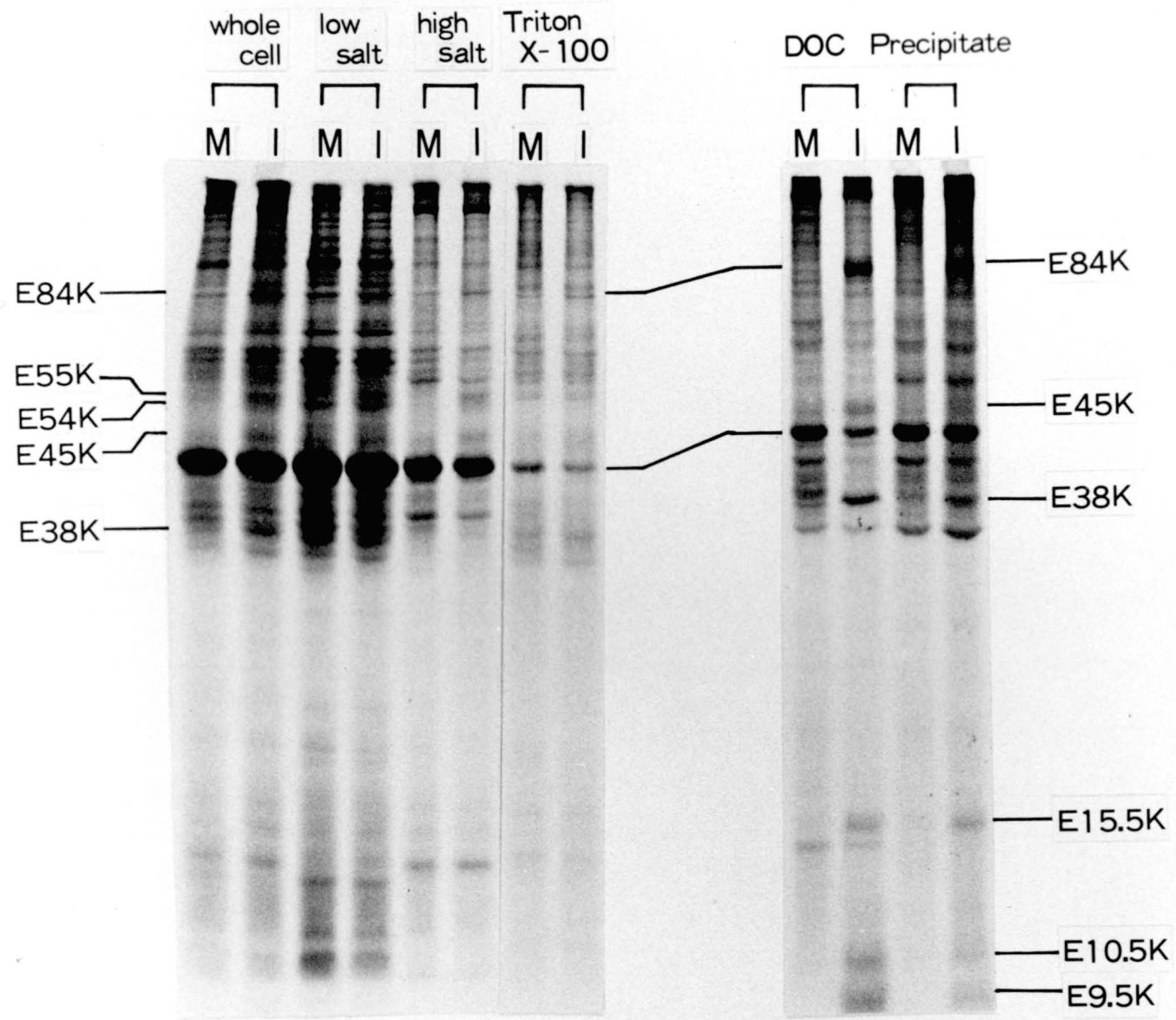
I, 4—5
M, 6—7
I, 6—7
M, 2—24
I, 2—24
E84K
E38K


whole cell
low salt
high salt
Triton X-100
DOC Precipitate
M
I
E84K
E55K
E54K
E45K
E38K
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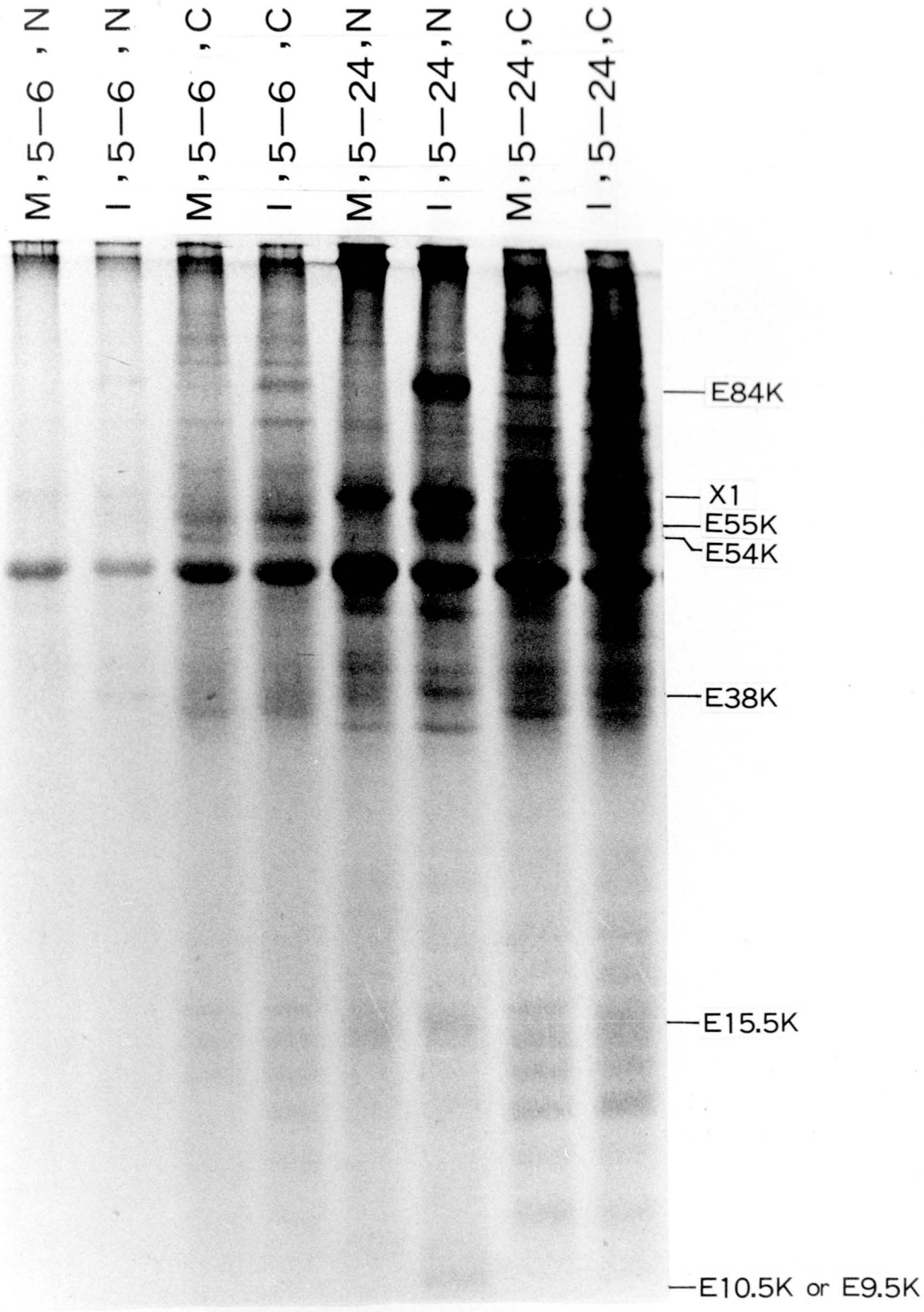
E38K
E15.5K
E10.5K
E9.5K

Fig 6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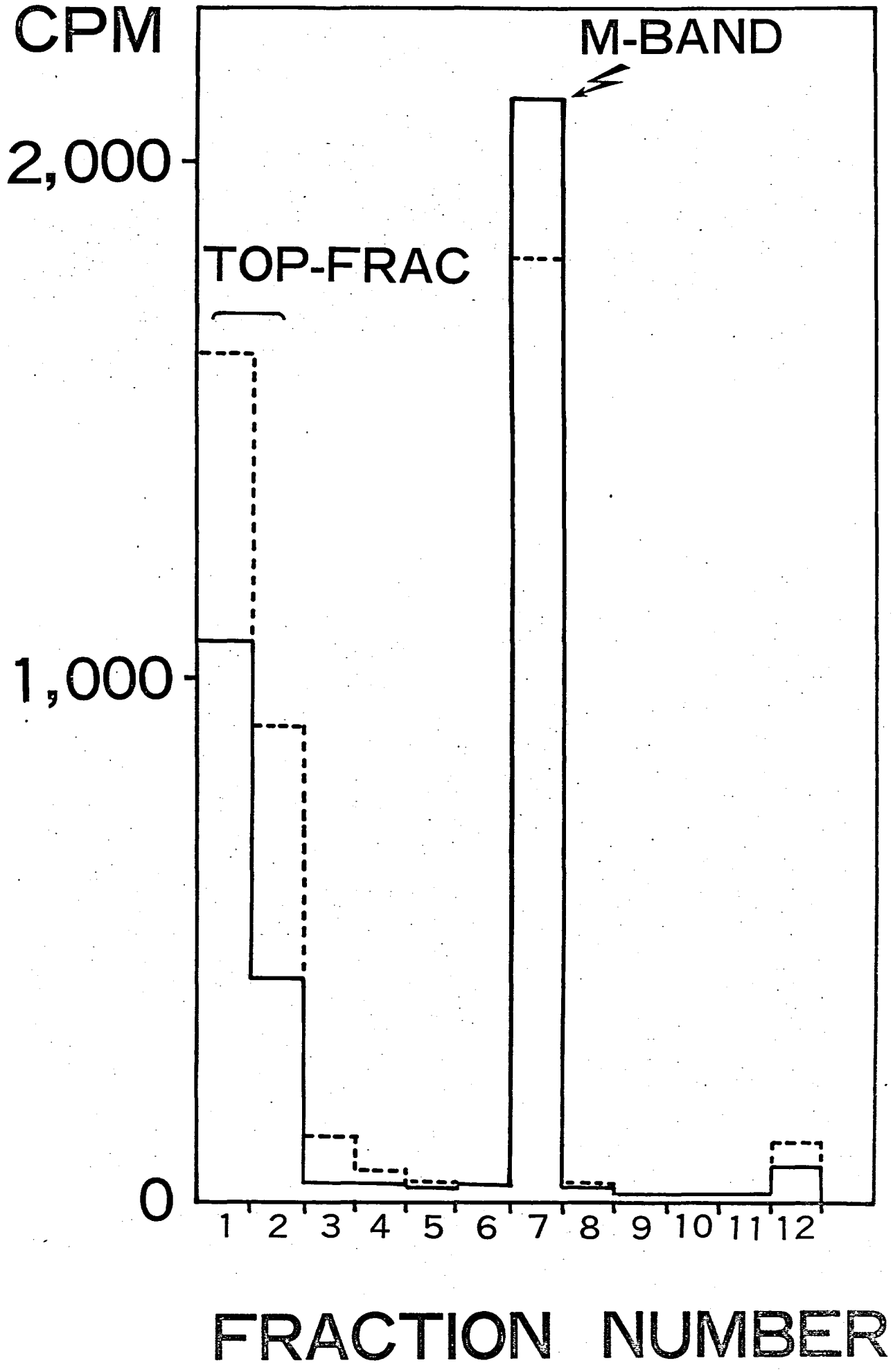
I,5—6,N
M,5—6,C
I,5—6,C
M,5—24,N
I,5—24,N
M,5—24,C
I,5—24,C
E84K
X1
E55K
E54K
E38K
E15.5K
E10.5K or E9.5K

Fig 7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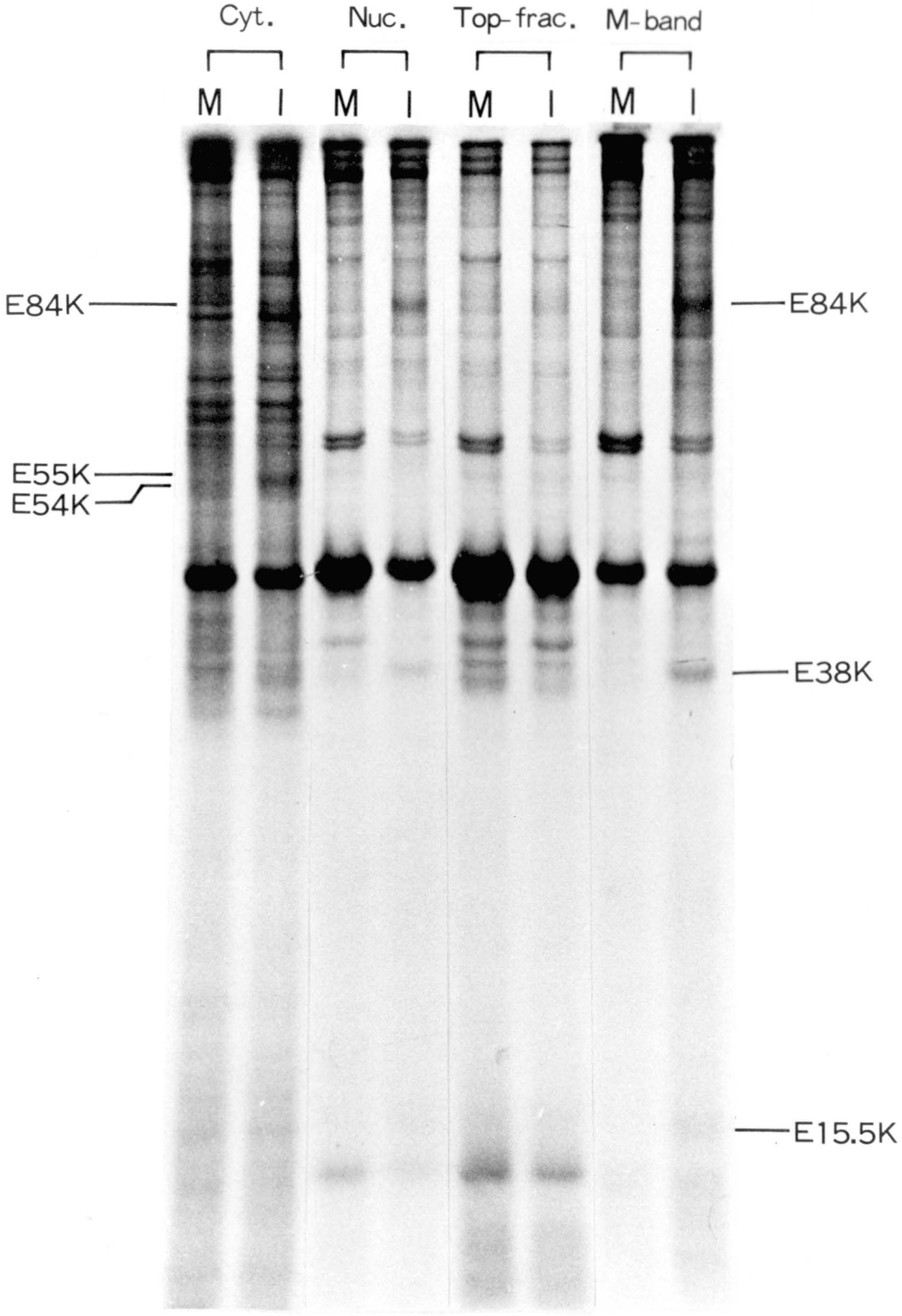
1,000
0
M-BAND
TOP-FRAC
1 2 3 4 5 6 7 8 9 10 11 12
FRACTION NUMBER


Cyt.
Nuc.
Top-frac.
M-band
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I
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M
I
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I
E84K
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E54K
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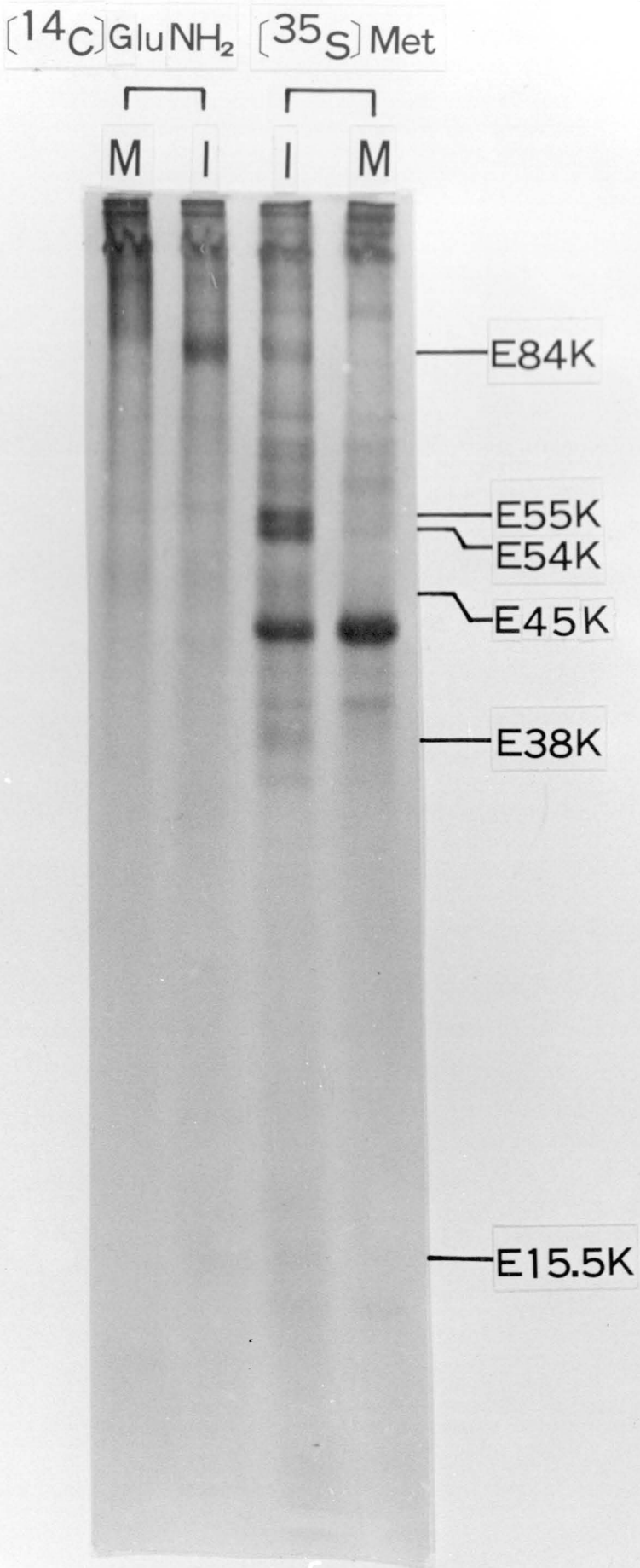
E38K
E15.5K

Fig 9



PART II: Preliminary Characterization of Temperature-sensitive Mutants of CELO Virus Defective in Viral Protein Production and Transportation.

ABSTRACT

Mono-specific antisera were prepared against two kinds of CELO-virus protein, i.e., hexon and L51K protein which are synthesized at the late stage of infection. By taking advantage of these sera for immunofluorescence microscopy, randomly selected 36 temperature-sensitive (ts) mutants which are defective in late infectious cycle were classified regarding the production and localization of the above two viral antigens: In the case of the hexon antigen, 9 mutants accumulated the antigen in the nucleus at the non-permissive temperature, 40°, as did the wild type virus (N type); 11 did not accumulate the antigen in the nucleus and retained it in the cytoplasm (C type); 8 had the antigen in the nucleus and the cytoplasm (CN type); 8 could not produce a detectable amount of the antigen. Concerning the localization of the L51K antigen, irrespective of the above grouping, 32 mutants (including 10 mutants out of 11 C-type mutants) accumulated the antigen in the nucleus; 3 had the antigen in the nucleus and the cytoplasm; and 1 (ts 10 of C-type mutants) could not produce a detectable amount of the antigen.

In regard to the retention of the hexon antigen, ts 8 and ts 10 of C-type mutants, being complementary each other as to the accumulation of the hexon antigen in the nucleus at 40°, were characterized more precisely. Ts 10 did not show any significant difference from the wild type concerning the cynthesis of viral polypeptides at 40° (including the hexon polypeptide) identified by SDS-polyacrylamide-gel-electrophoresis, the amount of the hexon antigen produced at 40°, extractability of the hexon antigen from infected cells, and the

sedimentation velocity of the hexon antigen. In contrast to ts 10, ts 8 was shown to produce an unexpectedly smaller amount of the hexon antigen at 40° under the same experimental condition, though this mutant was shown by immunofluorescence microscopy to retain the hexon antigen in the cytoplasm, immunofluorescence of which was roughly similar to that of ts 10.

INTRODUCTION

Temperature-sensitive conditionary lethal mutants (ts mutant) of human adenovirus (reviewed by Ginsberg and Yong, 1976) have been isolated in several laboraties in order to study the mechanisms of viral multiplication and the viurs-induced transformation. Chick embryo lethal orphan (CELO) virus, an avian adenovirus, was the first adenovirus from which a relatively large number of ts-mutants (49 ts-mutants) was isolated. They could grow at the permissive temperature, 31°, but not at the non-permissive temperature, 40° Ishibashi, 1970, 1971). These mutants were preliminarily classified into 5 groups on the basis of such properties as productivity of the viral antigen and transportability of the viral antigen from cytoplasm to nucleus. Eleven ts-mutants classified in the group III attracted our special attention: they could not transport the viral antigen at 40° from cytoplasm, the site of the viral antigen synthesis (Thomas and Green, 1966; Velicer and Ginsberg, 1968, 1970), to nucleus where the viral antigen assembled with the viral DNA to form virion (Boyer et al., 1959), but they produced the viral DNA and the virus-specific inclusion bodies in the nucleus as the wild type virus did. However, it was not known whether or not all the viral antigens were retained in the cytoplasm of cells infected at 40° with the group III mutants, or which one of the viral antigens was retained in the cytoplasm, since the antiserum used by Ishibashi (1970, 1971) to demonstrate the localization of the viral antigen by immunofluorescence microscopy technique was prepared against relatively crude extract of infected cells. In order to answer these questions, we prepared

two mono-specific antisera against individual viral proteins which had been elucidated and characterized in PART I; one was against the major virion protein, hexon, and the other was against the L51K protein. Applying these sera to immunofluorescence microscopy, we examined the localization of these two kinds of protein in cells infected with each one of the ts mutants at 40°. The immunofluorecence-microscopic study revealed that the hexon antigen was at least one of the antigen retained in the cytoplasm of cells infected with ts mutants of the group III at 40°. We examined, therefore, two representative mutants of this group (which, however, were found to belong to different complementation groups) in datail, regarding to the amount of the hexon antigen, its extractability from the infected cells, its sedimentation velocity, etc., by combining several biochemical and immunological techniques.

MATERIALS AND METHODS

Cell culture

Primary monolayer cultures of chicken kidney cells (CKC) were prepared, according to the procedures described in PART I.

Virus

The wild type CELO virus and its ts-mutants described by Ishibashi (1970) were used for the present experiments. Their infectivity was titrated by the plaque methods as described by Ishibashi (1971).

Preparation of antisera against viral antigens

i) Antiserum against hexon

The partially purifed hexon protein was obtained from the wild type virus-infected cell lysate by DEAE cellulose column chromatography as described by Maizel et al (1968), and mixed with the Freund's complete adjuvant. The mixture ~~was~~ injected into a rabbit at two week-intervals over a period of 8 weeks. The specificity of the antiserum thus obtained was determined by immunodiffusion and immunoprecipitation techniques described below.

ii) Antiserum against L51K protein

CELO virions (wild type) were purified from the infected CKC by the method described in PART I. A mixture of two volumes of purified virion solution and one volume of incomplete adjuvant (Crowle and Hu, 1966) was injected into a rabbit according to the procedures as described above. By using the immune serum thus

obtained, and infected and mock-infected cells which had been lysed in 5 mM Tris-HCl (pH 7.2) containing 1% SDS, immunodiffusion was carried out in 1% agarose containing 10 mM disodium ethylene-diamine-tetra-acetate (EDTA) and 25 mM veronal acetate (pH 8.6). Each one of infection-specific lines was cut out; washed extensively with a solution (0.85% NaCl, 10 mM EDTA, pH 7.5); and injected, after mixing with the incomplete adjuvant, into a rabbit according to the procedure as described above. The specificity of the antisera thus obtained was examined by immunodiffusion and immunoprecipitation as described below to obtain an antiserum specific for the L51K protein.

Examination of specificity of antisera

i) By immunodiffusion

Immunodiffusion was carried out in 1% agarose containing 10 mM EDTA and 25 mM veronal acetate (pH 8.6). For the test of an anti-hexon serum, the infected and mock-infected cells were lysed in phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) by freezing-thawing and used as antigens. For the test of an anti-L51K serum, they were lysed in 10 mM Tris-HCl (pH 7.2) containing 1% SDS and used as antigens. Detailed procedures for immunodiffusion test were described in the legend to Fig. 1.

ii) By immunoprecipitation

CKC (a total of 4.6×10^6 cells in two petri-dishes, each having a diameter of 90 mm) were infected with the wild type virus at a multiplicity of 100 plaque forming units (PFU) or mock-infected as described in PART I. The infected and mock-infected cells were

incubated with [^{14}C]leucine in 10 ml of the minimum essential medium (MEM) containing one-tenth the normal concentration of leucine plus 25 μCi [^{14}C]leucine (Daiichi Pure Chemicals, 280 mCi/mmole) and 2% dialyzed bovine serum between 18 and 30 hr after infection, in order to label virus-induced polypeptides (PART I). After labeling, the cells were washed once with PBS, scraped off from the glass surface of petri-dishes, and collected by low-speed centrifugation (250 x g, 10 min). All the procedures after labeling were performed at 0-4°. The pelleted cells were suspended in 3 ml of IM(III) buffer [0.5% sodium deoxycholate (DOC), 1% Triton X-100, 0.9% NaCl, 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), and sodium phosphate, pH 7.2] and disrupted with a tightly fitted teflon homogenizer. The resulting samples were clarified by centrifugation at 30,000 rpm (100,000 x g) for 2 hr in a Beckman SW50.1 rotor. With the supernatant as an antigen sample, the specificity of antisera was examined by means of the immunoprecipitation technique as described below.

Microscopic studies on infected cells

The preparation of specimens for microscopic studies was the same as that described previously (Ishibashi, 1971). For immunofluorescence staining with the anti-hexon serum, the direct method was used. Conjugation of fluorescein to γ-globulin and staining were carried out by following the method described by Ishibashi (1970). For immunofluorescence staining with the anti-L51K serum, the indirect method was employed. Infected cells on the cover-slip which had been fixed with cold acetone were exposed to the

unconjugated anti-L51K serum (4 units) for 30 min at 37°. Then, they were washed with PBS and exposed to fluorescein-conjugated goat γ-globulin against rabbit γ-globulin for 30 min at 37°. After the exposure to the second serum, they were washed again with PBS and used for microscopic examination.

Radioiotopic labeling of the virus-induced protein in wild type virus- or ts mutant-infected CKC

CKC, 5.5×10^5 cells per petri-dish (42 mm in diameter), were infected with the wild type virus, ts 8 or ts 10. The infected and mock-infected cells at 40° were lableled with [^{35}S]methionine for 3 hr from 22 hr after infection, and the cells at 31° were labeled for 9 hr from 56 hr after infection. The radioactive medium used in each petri-dish was 1.5 ml of methionine-free MEM plus 50 μCi [^{35}S]methionine (371 Ci/mmole, New England Nuclear). After labeling the cells were washed twice with PBS and stored at -20° until use.

Extraction of hexon antigen

The labeled cells were treated with IM(I) buffer (0.9% NaCl, 10 mM sodium phosphate, pH 7.2, 1 ml/petri-dish) and sonicated for 2 min in an ice-bath (9 KHz, 120 watt). The sample was centrifuged at 34,000 rpm (100,000 x g) in a Beckman type 40 rotor for 2 hr at 4° to separate the supernatant [IM(I)sup] from the pellet. The pellet was suspended into 0.5 ml of IM(I) buffer and sonicated under the same condition to disperse the pellet [IM(I)ppt]. The sample was mixed with 0.5 ml of IM(II) buffer (0.9% NaCl, 2% Triton X-100,

1% DOC, 2 mM PMSF, and 10 mM sodium phosphate, pH 7.2), and centrifuged under the same condition as described above to separate the supernatant [IM(II)sup] from the pellet [IM(II)ppt]. These fractions thus obtained were used for complement-fixation (CF) test and immunoprecipitation test to determine the amount of the hexon antigen present in the fractions. Aliquots of IM(I)sup and IM(I)ppt fractions were used for CF test. For immunoprecipitation study, IM(I)sup, which was mixed with an equal volume of IM(II) buffer prior to immunoprecipitation, and IM(II)sup were used.

Sucrose density gradient centrifugation

Buffer used for sucrose density gradient contained 0.9% NaCl, 1 mM PMSF, and 10 mM sodium phosphate (pH 7.2). IM(I)sup (0.35 ml) was layered on top of a sucrose density gradient from 5 to 20% with a total volume of 4.7 ml, and centrifuged at 4° for 14 hr at 30,000 rpm in a Beckman SW50.1 rotor. The gradient was fractionated into 22 fractions by piercing through the bottom of the centrifuge tube. Each fraction was divided into 3 portions: one was used for determination of trichloroacetic acid (TCA)-insoluble radioactivity present in each fraction; another, for the virus-induced polypeptide analysis by SDS-PAGE; and the remaining was mixed with an equal volume of IM(II) buffer and subjected to immunoprecipitation, followed by SDS-PAGE, in order to determine the amount of the hexon antigen in each fraction.

Immunoprecipitation technique

In a pilot experiment, the optimal ratio of volumes of the rabbit serum to the goat anti-rabbit γ-globulin serum was determined. Subsequently, each 0.2-ml aliquot of the antigen sample was mixed with 5 μl of various rabbit antisera against viral antigen(s) and incubated for 16 hr at 4°. The resulting sample was mixed with 120 μl of the goat anti-rabbit γ-globulinserum and incubated for additional 3 hr at 4°. After the incubation, the sample was mixed with 4 ml of IM(III) buffer and centrifuged (12,000 x g, 15 min, 4°) and the supernatant was discarded. The pellet was washed two more times with IM(III) buffer and once with 10 mM Tris-HCl (pH 8.0) by the centrifugation. Then, the pellet was dissolved in the SDS-sample buffer described by Maizel (1971) and analyzed by SDS-PAGE.

Complement-fixation test (CF test) technique

The complement-fixation test was conducted as described by Nishioka and Okada (1966).

SDS-polyacrylamide gel electrophresis (SDS-PAGE) and scintillation autoradiogram

SDS-PAGE was carried out as described in PART I. After electrophoresis the gel was stained with Coomasie Brilliant Blue R-250, then treated with 20% (W/W) 2,5-diphenyl oxazole (PPO) (Bonner and Laskey, 1974), and dried on filter paper. The dried gel was kept contact with X-ray film (Kodak RP-Royal X-Omat film) at -70° to visualize a radioactive band(s).

RESULTS

Specificity of antisera against viral protein

i) Anti-hexon serum

Specificity of the anti-hexon serum was examined by two different techniques (immunodiffusion and immunoprecipitation). Immunodiffusion gave a single precipitation line between the anti-hexon serum and infected sample, and gave no line between the anti-hexon serum and mock-infected sample (Fig. 1A). Immunoprecipitation of the anti-hexon serum with ^{14}C-labeled infected or mock-infected sample revealed that the amount of radioactivity of the immunoprecipitated materials formed with the infected sample was 10-fold higher than that with the mock-infected sample (Table 1); the precipitate with the infected sample gave one band (corresponding to hexon polypeptide band on the basis of mobility in SDS-PAGE), and the precipitate with the mock-infected sample gave no distinct band (Fig. 2). When immunoprecipitation was carried out between the antiserum against bovine serum and the antigen samples, the amount of the radio-activity precipitated showed no difference between the infected and the mock-infected samples, and was similar to that obtained between the anti-hexon serum and the mock-infected sample. These precipitates showed no distinct band (data not shown) in SDS-PAGE. These results indicated that the anti-hexon serum prepared was specific for the hexon protein.

ii) Anti-L51K serum

Similarly, the specificity of the anti-L51K serum was examined with immunodiffusion and immunoprecipitation. As shown in Fig 1B, a single line was observed only between the antiserum and infected sample when the immunodiffusion was conducted between the antiserum and infected or mock-infected sample. In the immunoprecipitation study, the amount of radioactive precipitate formed with the ^{14}C-labeled infected sample was 5-fold higher than that with the ^{14}C-labeled mock-infected sample (Table 1), and only the precipitate formed between the antiserum and the infected sample gave one distinct band in SDS-PAGE (Fig 2), which corresponded to the L51K polypeptide band on the basis of the electrophoretic mobility. The results indicated that the anti-L51K serum was specific for L51K protein. The specificity of the antisera for proteins they recognized was further confirmed by the immunodiffusion test, in which the precipitin line formed between the anti-hexon and infected cell lysate did not fuse the line formed between the anti-L51K serum and infected cell lysate (Fig. 1C).

Localization of hexon and L51K antigens in cells infected with various ts mutants at the non-permissive temperature (40°)

Cover-slip cultures of CKC were infected with the wild type virus of individual ts mutants available. After 32-hr incubation at 40°, the cultures were tested for localization of viral antigens by

immunofluorescence staining microscopy with the anti-hexon and the anti-L51K serum. Findings are summarized in Table 2. Percentage of infected cells judged from immunofluorescence staining with the antisera was similar to that of the inclusion body-positive cells demonstrated by hematoxylin-eosin staining (data not shown). Briefly, the findings with the anti-hexon serum were almost the same as those with the anti-CELO virus serum reported prevously (Ishibashi, 1970, 1971). Nine out of 36 mutants tested [these 9 belong to the group I (Ishibashi, 1971)] accumulated the antigen in the nucleus; 11 mutants [11, to the group III (Ishibashi, 1971)] retained the antigen in the cytoplasm; 8 mutants [5, to the group II (Ishibashi, 1971); 3, to the group I] had the antigen both in the cytoplasm and the nucleus; and 8 mutants [8, to the group IV (Ishibashi, 1971)] could not produce a detectable amount of the antigen. In the case of the immunofluorescence staining with the anti-L51K serum, the results were quite different from those obtained with the anti-hexon serum. Thirty-two mutants (11 belong to the group I; 5, to the group II; 10, to the group III; 6, to the group IV); 3 mutants (1, to the group I; 2, to the group IV); and 1 mutant (the group III) could not produce a detectable amount of the antigen. These results indicated that, in regard to the group III mutants, the mutants retained at least the hexon antigen in the cytoplasm at 40°.

Production of viral polypeptides in ts 8- or ts 10-infected cells

As to ts 8 and ts 10 belonging to the group III, more precise biochemical analyses were undertaken, since immunofluorescence studies showed that these mutants complemented each other regarding

nuclear accumulation of the viral antigen (Ishibashi, 1970) or the hexon antigen (Yasue, unpublished data).

CKC were infected with the wild type virus, ts 8, or ts 10 at 40° and 31° as described in MATERIALS AND METHODS. In a parallel experiment it was confirmed by immunofluorescence staining with the anti-hexon serum that 30 to 50% of cells were infected under this condition (Fig. 3). The infected and mock-infected cells at 40° were labeled with [^{35}S]methionine for 3 hr from 22 hr after infection, and the cells at 31° were labeled for 9 hr from 56 hr after infection. A portion of the labeled cells was treated with TCA and analyzed by SDS-PAGE to examine the production of viral polypeptides. When electropherograms of ^{35}S-polypeptides of cells infected at 40° were compared (Fig. 4), the viral polypeptide bands (L76K, L54.5K, L51K, E38K, and L32K) (PART I) were detected equally well in the cells infected with the wild type virus, ts 8, or ts 10, though other virus-induced polypeptide bands were not detected, possibly due to the low multiplicity of infection. The hexon polypeptide band, however, was hardly detected in cells infected with ts 8, while the band was detected in cells infected with the wild type virus or ts 10. On the other hand, at 31°-incubation, no difference was observed among the virus strains concerning the production of the virus-induced polypeptides including the hexon polypeptide.

Extraction of the hexon antigen from infection cells

In order to compare ts 8- or ts 10-hexon antigen produced with the wild type-hexon antigen regarding extractability from the infected

cells and sedimentation velosity in a sucrose density gradient centrifugation, the remaining portion of the labeled cells described in the previous section was fractionated by the procedure described in MATERIALS AND METHODS. The percentage of the TCA-insoluble radioactivity found in each fraction is given in Table 3; the distribution of radioactivity in each fraction was irrespective of the incubation temperatures and the viral strains used. The recovery of the hexon antigen was determined by CF test with the anti-hexon serum (Table 4). Approximately, 30 to 70% of the hexon antigen present in cells infected with the wild type virus or ts 10 at both temperatures appeared to be recovered in the IM(I)sup fraction. In the case of ts 8-infection at 40°, however, the hexon antigen could not be detected in the fractionated sample and even in the whole cell lysate, though the hexon antigen was recovered from the cells infected with ts 8 at 31° as from the cells infected with the wild type virus or ts 10. Immunoprecipitation with the anti-hexon serum (Table 3) revealed that the radioactivity of the precipitates in IM(I)sup and in IM(II)sup showed no difference among the viral strains at 31°. But at 40° the radioactivity of the precipitates of IM(I)sup and IM(II)sup of the ts 8-infected cells were much smaller than those obtained from the wild type virus- and the ts 10-infected samples, and were little more than those from the mock-infected samples. The specificity of the immunoprecipitation was confirmed by analyzing the precipitates with SDS-PAGE. As shown in Fig. 5, the precipitated radioactive materials of the wild type virus- and the ts 10-infected samples at both temperatures and of the ts 8-infected

sample at 31° mainly consisted of the hexon polypeptide; but, that of the ts 8-infected sample at 40° consisted of small amount of the hexon polypeptide and very small amount of unidentified polypeptides. No radioactive polypeptide was detected in the precipitates of the mock-infected samples at both temperatures. The results of immunoprecipitation were accordant with those of SDS-PAGE of the whole infected cell lysate (Fig. 4) and of the CF test (Table 4).

Sucrose density gradient centrifugation analysis

As an attempt to distinguish the ts 10-hexon antigen produced at 40° from the wild type-hexon antigen, sucrose density gradient centrifugation of the ts 10-hexon antigen and the wild type-hexon antigen was carried out.

IM(I)sup fractions of the cells infected with the wild type virus or ts 10 at 40° were layered on top of the sucrose density gradient (5-20%, W/V), and subjected to centrifugation under the condition as described in MATERIALS AND METHODS. As a control, the fraction of the mock-infected sample was treated similarly. The sedimentation patterns of the IM(I)sup of the wild type virus- and the ts 10-infected samples (Fig. 6) revealed two major peaks of TCA-insoluble radioactivity: one was at 13 S (designated as 13 S peak); the other, at 4 S (designated 4 S peak). The sedimentation pattern of IM(I)sup of the mock-infected sample (Fig. 6) revealed only one major peak corresponding to 4 S peak. Aliquots of fractions of the gradients were TCA-precipitated and analyzed by SDS-PAGE. Another aliquots were analyzed by immunoprecipitation. As shown

in Fig. 6, when immunoprecipitated, the radioactivity in each fraction of the wild type virus- and the ts 10-infected samples formed one major peak corresponding to 13 S peak, and that of the mock-infected sample formed no distinct peak. The immunoprecipitated radioacitve materials of each fraction of the wild type virus-, the ts 10-, and the mock-infected samples were analyzed by SDS-PAGE. As shown in Fig. 7, the radioactive materials of 13 S peak consisted only of the hexon polypeptide, indicating that even at 40° ts 10-produced hexon was trimer of the hexon polypeptide as was the wild type-hexon. The remaining aliquots of fractions of the sucrose density gradients were treated with TCA and analyzed by SDS-PAGE (Fig. 7). The hexon polypeptide of ts 10 was detected only in the fraction of 13 S peak as that of the wild type virus was.

DISCUSSION

We prepared monospecific antisera against the hexon and the L51K protein of CELO virus (wild type). The hexon is the major protein, occupying 240 capsomeres out of 252 capsomeres which locate at the surface of the icosahedral CELO virion. L51K protein is one of the 15 virus-induced late proteins we detected previously (PART I). Since we observed the presence of one protein (V51K) in the virion which showed the same mobility with the L51K in SDS-PAGE, L51K protein seemed to take part in construction of the CELO virion. Applying those mono-specific antisera to immunofluorescence microscopy, we examined the localization of the hexon antigen and the L51K antigen in cells infected with various kinds of ts mutants of CELO virus (Ishibashi, 1970, 1971) at the non-permissive temperature (40°). Experimental results obtained with regard to the localization of the hexon antigen were quite similar to those obtained with the antiserum prepared with relatively crude virus preparation (Table 2) (Ishibashi, 1970, 1971). The temperature-sensitive mutants belonging to the group I of the previous classification (Ishibashi, 1971) accumulated the hexon antigen in the nucleus even at 40° as the wild type did though 3 mutants of this group showed the same phenotype of the group II mutants. The group III mutants retained the hexon antigen in the cytoplasm at 40°. The group II showed intermediate phenotype between the group I and III. The group IV did not produce a detectable amount of the hexon antigen. On the other hand results obtained regarding the localization of the L51K antigen were contrastive to those obtained for the hexon antigen. The mutants belonging to

the above 4 groups accumulated the L51K antigen in the nucleus at 40° as the wild type virus though 3 mutants (one in the group I, two in the group IV) ahd the antigen both in the cytoplasm and the nucleus. One exceptional case was ts 10 which could not produce the antigen at 40° (though it produced L51K polypeptide as much as the wild type virus). The transportation of the hexon antigen did not coordinate with that of the L51K antigen in the cells infected with various ts mutants. Further there was no correlation between the production the hexon antigen and the L51K antigen. With respect to the group III mutants, the hexon antigen at 40° was retained in the cytoplasm though the L51K antigen detected was accumulated in the nucleus. Therefore we could eliminate a possibility that transportation of all the viral proteins from the cytoplasm to the nucleus was paralyzed when the hexon, the major viral protein, could not be transported. It is, however still required to answer such question as whether or not the group III mutants retain viral proteins other than the hexon antigen in the cytoplasm at 40°, by extending the present type of experiments, since we tested the localization for only two viral proteins out of more than 10 in the above experiments.

Combining immunological and biochemical techniques, we examined, the nature of the hexon antigen produced by ts 8 and ts 10 of the group III, which complemented each other regarding the the accumulation of the hexon antigen in the nucleus. The hexon antigen produced at 40° by one of the ts mutants, ts 10, did not show any significant difference from that of the wild type virus with respect to the following points; 1) the amount of hexon polypeptide synthesis, 2) extractability of the hexon antigen (CF test) (Table 3), 3) amount of the hexon antigen

detected with CF test or immunoprecipitation test, 4) sedimentation velocity of the hexon antigen demonstrated by sucrose density gradient centrifugation. Results similar to ts 10 reported here were obtained with the only one ts mutants so far tested in human adenovirus which showed cytoplasmic retention of the hexon antigen (Kauffman and Ginsberg, 1976). More extensive analysis must be done in order to answer the following questions: whether the difference in conformation of the ts 10 hexon antigen at 40° from that of the wild type virus, which could not be detected in the above experiments, is responsible for the difference in transport of the hexon antigen, or whether a hypothetical viral protein essential for transportation of hexon antigen is defective in ts 10. Based on the hypothesis that there is a viral protein essential for transport of the hexon antigen, and that ts 10 is defective in one gene, L51K antigen may play a role in transportation of the hexon antigen, which is deduced from the fact that ts 10 does not produce the immunological reactive L51K antigen (Table 2).

We failed to demonstrate the presence of the hexon antigen of ts 8 at 40° in CF test (Table 4) and failed to detect the hexon polypeptide by SDS-PAGE analysis (Fig. 4) as well, though we detected very small amount of the hexon antigen in immunoprecipitation (Table 3 and Fig. 5). It seems very difficult to reconcile these results with those obtained by immunofluorescence staining technique. It was shown that the number of immunofluorescence-positive ts 8-infected cells was roughly equal to that of ts 10-infected cells, and that the intensity of immunofluorescence of the hexon antigen in the cytoplasm of the ts 8-infected cells at 40° could not be distinguished from that of ts 10. If we assume that the direct method of immunofluorescence staining is

more sensitive than other methods employed, the above discrepancy could be reconciled, though it seems rather an unlikely explanation. Therefore it is worth doing to fix the ts 8-infected cells at 40° *in situ* with cold acetone as in the case of treatment for immunofluorescence staining, prior to the analysis of the aceton-fixed antigen by CF test and radioimmunoassay. As an another experiment, it is necessary to solubilize the hexon antigen by a rather mild method such as freezing-thawing of the infected cells for subsequent analysis of the antigen. Since the period of pulse-labeling with a radioactive amino acid in the present study was relatively long (3 hr), a very short pulse and chase experiment is required in order to examine the possible fate of the ts 8-hexon polypeptide after translation on cytoplasmic ribosomes.

We are attempting the peptide mapping studies with the tryptic digests of the hexon obtained from cells infected with ts 8, ts 10 or the wild type virus at 31° in order to see whether the hexon of mutants has a different primary structure from the wild type hexon.

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Legend to Fig. 1. Immunodiffusion between antisera against viral proteins and infected or mock-infected cell lysates. Immunodiffusion was conducted in 1% agarose containing 10 mM EDTA and 25 mM veronal acetate (pH 8.6). Diameter of well was 3 mm and distance between the centers of 2 wells was 7 mm. A, examination of specificity of the anti-hexon serum; B, examination of specificity fo the anti-L51K serum; C, relationship between the anti-hexon serum and the anti-L51K serum. a_1, the wild type virus-infected cells lysed in PBS by freezing-thawing; a_2, mock-infected cells treated as in a_1; a_3, the wild type virus-infected cells lysed in 10 mM Tris-HCl (pH 7.2) containing 1% SDS; a_4, mock-infected cells treated as in a_3. S_1, the anti-hexon serum; s_2, the anti-L51K serum.

Lengend to Fig. 2. Examination of specificity of the antisera by immunoprecipitation and subsequent SDS-PAGE. CKC (4.6×10^6 cells) were infected with the wild type virus at a multiplicity of 100 PFU and labeled with [^{14}C]leucine between 18 and 30 hr after infection (see text). The labeled cells were suspended into 3 ml of IM (III) buffer and disrupted with a tightly fitted teflon homogenizer. The resulting sample was clarified by centrifugation at 30,000 rpm (100,000 x g) for 2 hr in a Beckman SW50. 1 rotor. A 0.2-ml aliquot of the supernatant were mixed with 5 µl of individual antisera, and processed by following the immunoprecipitation technique described in the text. The immunoprecipitated materials were subjected to SDS-PAGE, followed by autoradiography. A, mock-infected cells. B, infected cells. C, immunoprecipitated materials formed between the anti-L51K serum and the infected

sample. D, immunoprecipitated materials formed between the anti-L51K serum and the mock-infected sample. E, immunoprecipitated materials formed between the anti-hexon serum and the mock-infected sample. F, immunoprecipitated materials formed between the anti-hexon serum and the infected sample.

Legend to Fig. 3. Immunofluorescence staining with anti-hexon serum. CKC were infected with the wild type virus, ts 8 or ts 10 as described in the text. After 32-hr incubation at 40°, the cultures were treated as described in Materials and Methods for direct immunofluorescence staining with the anti-hexon serum. A, the wild type virus. B, ts 8. C, ts 10.

Legend to Fig. 4. Virus-induced polypeptides present in cells infected with the wild type virus, ts 8, or ts 10 at 40° and 31°. CKC, 5×10^5 cells per petri-dish (42 mm in diameter) were infected with the wild type virus, ts 8, or ts 10. The percentage of infected cells were monitored by immunofluorescence staining, which had been shown in Fig. 3. The infected and mock-infected cells at 40° were labeled with [^{35}S]-methionine for 3 hr from 22 hr after infection, and the cells at 31° were labeled for 9 hr from 56 hr after infection. The radioactive medium used in each petri-dish was 1.5 ml of methionine free MEM plus 50 µCi [^{35}S]methionine (371 Ci/mmole, New England Nuclear). After labeling, a portion of the cells were analyzed with SDS-PAGE, followed by autoradiography.

Legend to Fig. 5. SDS-PAGE analysis of immunoprecipitated materials from IM(I)sup and IM(II)sup of the cells infected with various strains at 40° and 31°. IM(I)sup and IM(II)sup were prepared from the cells shown in Fig. 3, and applied to immunoprecipitation as described in the text. The immunoprecipitated materials were subjected to SDS-PAGE, followed by autoradiography.

Legend to Fig. 6. Sucrose density gradient centrifugation of the hexon antigen. The IM(I)sup (0.35 ml) of the wild type virus-, ts 10-, and mock-infected samples at 40° shown in Fig. 5 was layered on the top of a sucrose density gradient centrifugation from 5 to 20% (W/V) with a total volume of 4.7 ml, and centrifuged at 4° for 14 hr at 30,000 rpm in a Beckman SW50.1 rotor. [The buffer used for the sucrose density gradient contained 0.9% NaCl, 1 mM PMSF, 10 mM sodium phosphate (pH 7.2)] After centrifugation, the gradient was fractionated by piercing through the bottom of the centrifuge tube. Aliquots of each fraction were used for determination of TCA-insoluble radioactivity (A, B, C) and for immunoprecipitation (D, E, F). The wild type virus-infected sample, A and D; ts 10-infected sample, B and E; mock-infected sample, C and F.

Legend to Fig. 7. SDS-PAGE analysis of the samples fractionated by sucrose density gradient centrifugation. TCA-insoluble (A, B, C) and immunoprecipitated (D, E)materials described in the legend to Fig. 6. were subjected to SDS-PAGE, followed by autoradiography. The wild virus-infected sample, A and B; ts 10 infected sample, B and E; mock-infected sample, C.

legend to Table 1 CKC were infected with the wild type virus at a multiplicity of 100 PFU or mock-infected, and labeled with [^{14}C]leucine at 40° between 18 to 30 hr after infection. Labeled cells were lysed in IM(III) buffer by sonication as described in MATERIALS AND METHODS. The resulting samples were clarified by centrifugation (100,000 x g, 2 hr, 4°), and the supernatant was used as antigen for immunoprecipitation to examine the specificity of the antisera. Numbers in the talbe show radioactive counts obtained by immunoprecipitation.

legend to Table 2 Temperature-sensitive mutants are listed up following the previous classification (Ishibashi, 1971) which was based on the immunofluorescence-microscopic observation of the viral antigen with the antiserum prepared against relatively crude CELO virus preparation; namely, at 40°, Group I mutants accumulated the viral antigen in the nucleus, Group II mutants had the antigen both in the nucleus and the cytoplasm, Group III mutants retained the antigen in the cyoplasm, and Group IV mutants did not produce a detectable amount of the antigen.

The localization of the hexon or the L51K antigen was indicated by following symbols: N, in the nucleus; CN, both in the nucleus and in the cytoplasm; C, in the cytoplasm; (-), not detected.

* Relative ratio of the amount of the viral antigen in the cytoplasm to in the nucleus was greater than other mutants of the group II.

** The amount of the viral antigen was smaller than that of other mutants of the group III.

legend to Table 3 CKC were infected with the wild type virus, ts 8, or ts 10. The infected cells at 40° were labeled with [^{35}S]methionine for 3 hr from 22 hr after infection, and the cells at 31° were labeled for 9 hr from 56 hr after infection. The mock-infected CKC were treated as in the infected cells. The labeled cells were suspended into IM(I) buffer (0.9% NaCl, 10 mM sodium phosphate, pH 7.2) and sonicated for 2 min in an ice-bath (9 KHz, 120 watt). The sample was centrifuged at 34,000 rpm (100,000 x g) in a Beckman type 40 rotor for 2 hr at 4° to separate the supernatant [IM(I)sup] from the pellet. The pellet was suspended into IM(I) buffer and sonicated under the same condition to disperse the pellet [IM(I)ppt]. The sample was mixed with an equal volume of IM(II) buffer (0.9% NaCl, 2% Triton X-100, 1% DOC, 2 mM PMSF, and 10 mM sodium phosphate, pH 7.2), and centrifuged under the same condition as described above to separate the supernatant [IM(II)sup] from the pellet [IM(II)ppt]. An aliquot of each fraction was used for determination of TCA-insoluble radioactivity and for immunoprecipitation.
Abbreviation: A, percentage distribution of TCA-insoluble ^{35}S-materials; B, the ratio of immunoprecipitated ^{35}S-hexon antigen to the TCA-insoluble ^{35}S-materials of the corresponding fraction is given in percentage.

* Total TCA-insoluble radioactivity (cpm) present in the fraction.

legend to Table 4 The fractionated samples shown in Table 3 were subjected to CF test with the anti-hexon serum to determine the amount of the hexon antigen in each fraction.

* The numbers represent the reciprocals of maximal dilution of samples to give positive CF reaction.

Table 1 Examination of specificity of antisera by immunoprecipitation

Antiserum	Infected sample (input, 267,400 cpm)	Mock-infected sample (input, 322,000 cpm)
anti-hexon	20,460 cpm	1,890 cpm
anti-L51K	7,080	1,350
anti-bovine serum	2,850	2,490

Table 2 Immunofluorescence staining of ts mutant-infected cells with antisera against hexon and L51K protein.

	localization of viral antigen			localization of viral antigen	
ts-strain	hexon	L51K	ts-strain	hexon	L51K
group I			group III		
ts 7	N	N	ts 5	C	N
ts 12	N	N	ts 8	C	N
ts 16	N	N	ts 10	C	(-)
ts 24	N	CN	ts 13	C	N
ts 31	N	N	ts 19	C	N
ts 32	CN	N	ts 20	C	N
ts 33	N	N	ts 28	C	N
ts 34	N	N	ts 29	C	N
ts 36	CN	N	ts 30	C	N
ts 41	N	N	ts 37	C	N
ts 43	CN	N	ts 49	C**	N
ts 46	N	N			
			group IV		
group II			ts 2	(-)	CN
ts 1	CN	N	ts 4	(-)	N
ts 35	CN	N	ts 6	(-)	N
ts 39	CN	N	ts 11	(-)	N
ts 48	CN	N	ts 14	(-)	N
ts 50	CN*	N	ts 15	(-)	N
			ts 17	(-)	N
			ts 23	(-)	CN

Table 3 Distribution of TCA-insoluble ^{35}S-materials in fractionated samples and the amount of ^{35}S-hexon antigen in individual fractions

infection at 40°	Wild type virus (A)	Wild type virus (B)	ts 8 (A)	ts 8 (B)	ts 10 (A)	ts 10 (B)	mock (A)	mock (B)
Whole cell	100% (5.32×10^6)*		100% (6.07×10^6)*		100% (6.67×10^6)*		100% (4.41×10^6)*	
IM(I)sup	43	6.9	46	2.7	46	5.2	42	1.7
IM(II)sup	32	10.2	27	2.1	31	4.4	23	0.3
IM(II)ppt	25		27		23		35	
Infection at 31°								
Whole cell	100 (1.65×10^7)*		100 (1.52×10^7)*		100 (1.78×10^7)*		100 (1.74×10^7)*	
IM(I)sup	43	7.9	44	7.1	47	8.7	44	1.8
IM(II)sup	31	7.3	17	4.1	24	8.0	29	0.5
IM(II)ppt	26		39		29		27	

Table 4 Test for amount of hexon antigen present in the fractionated samples by complement-fixation.

	wild type virus.		ts 8		ts 10		mock	
	40°	31°	40°	31°	40°	31°	40°	31°
Whole cell	128	32	<2	32	32	64	<2	<2
IM(I)sup	16	16	<2	16	16	8	<2	<2
IM(I)ppt	16	8	<2	4	8	8	<2	<2

Fig I

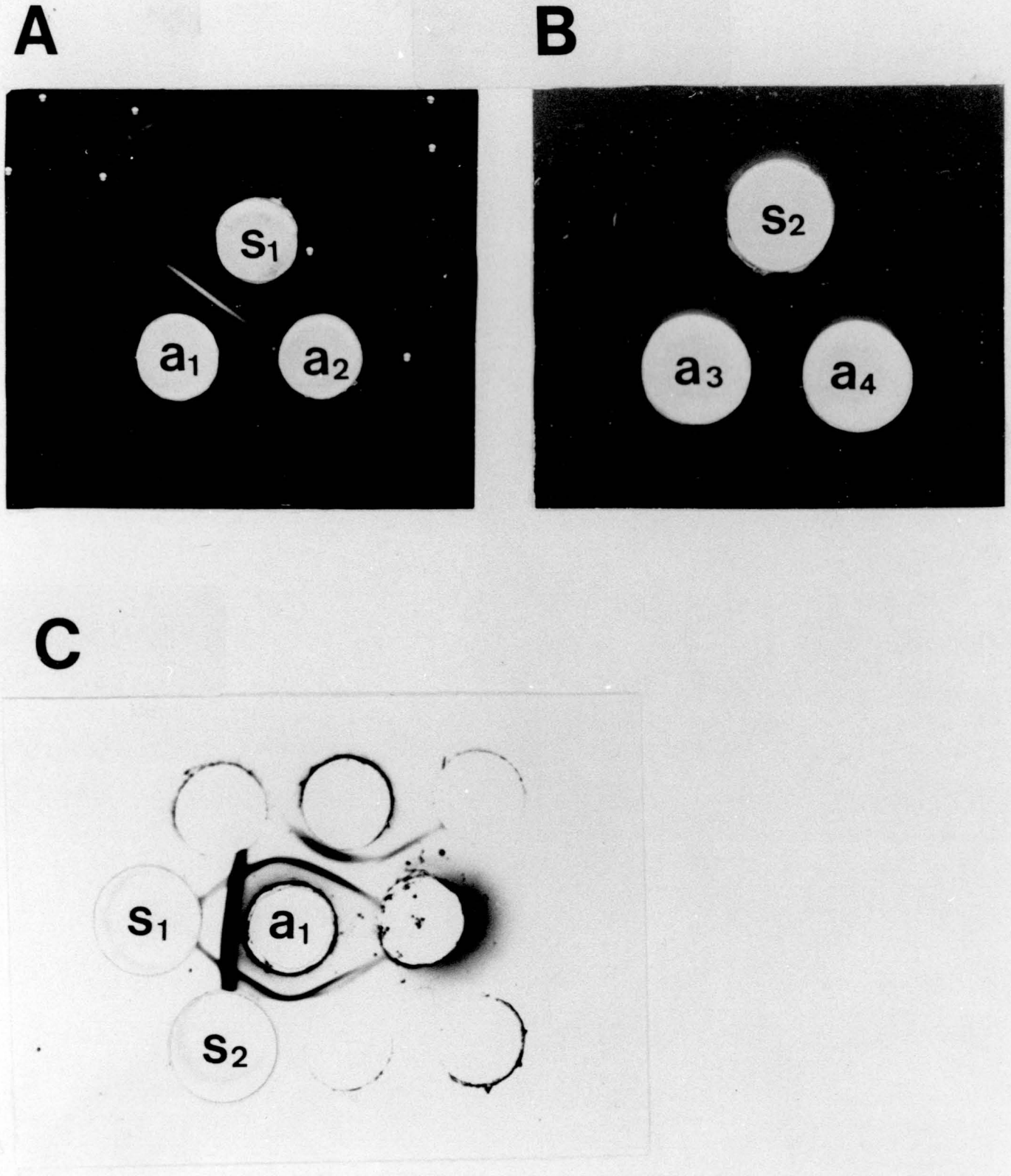


Fig 2

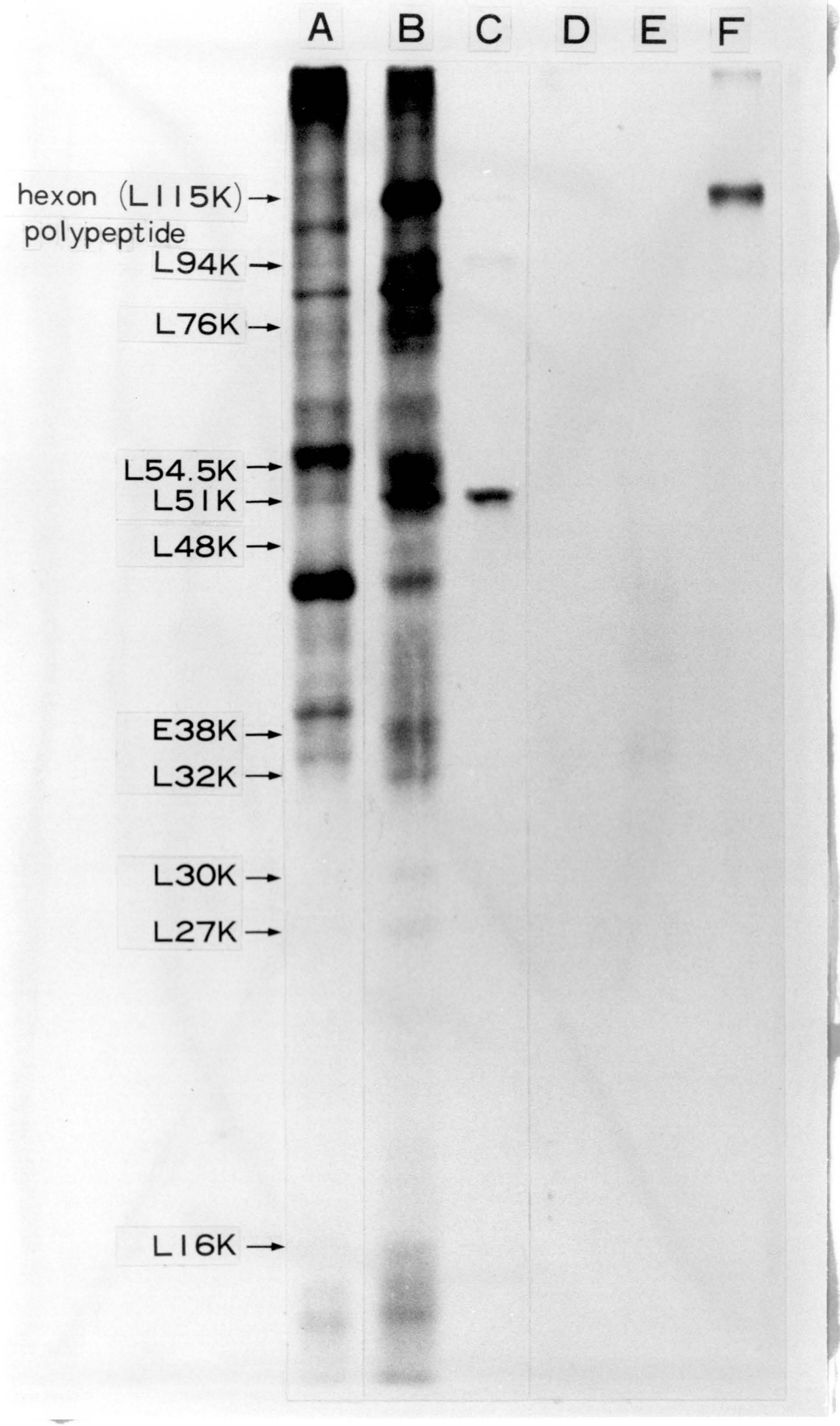

A
B
C
D
E
F
hexon (L115K)
polypeptide
L94K
L76K
L54.5K
L51K
L48K
E38K
L32K
L30K
L27K
L16K

Fig 3

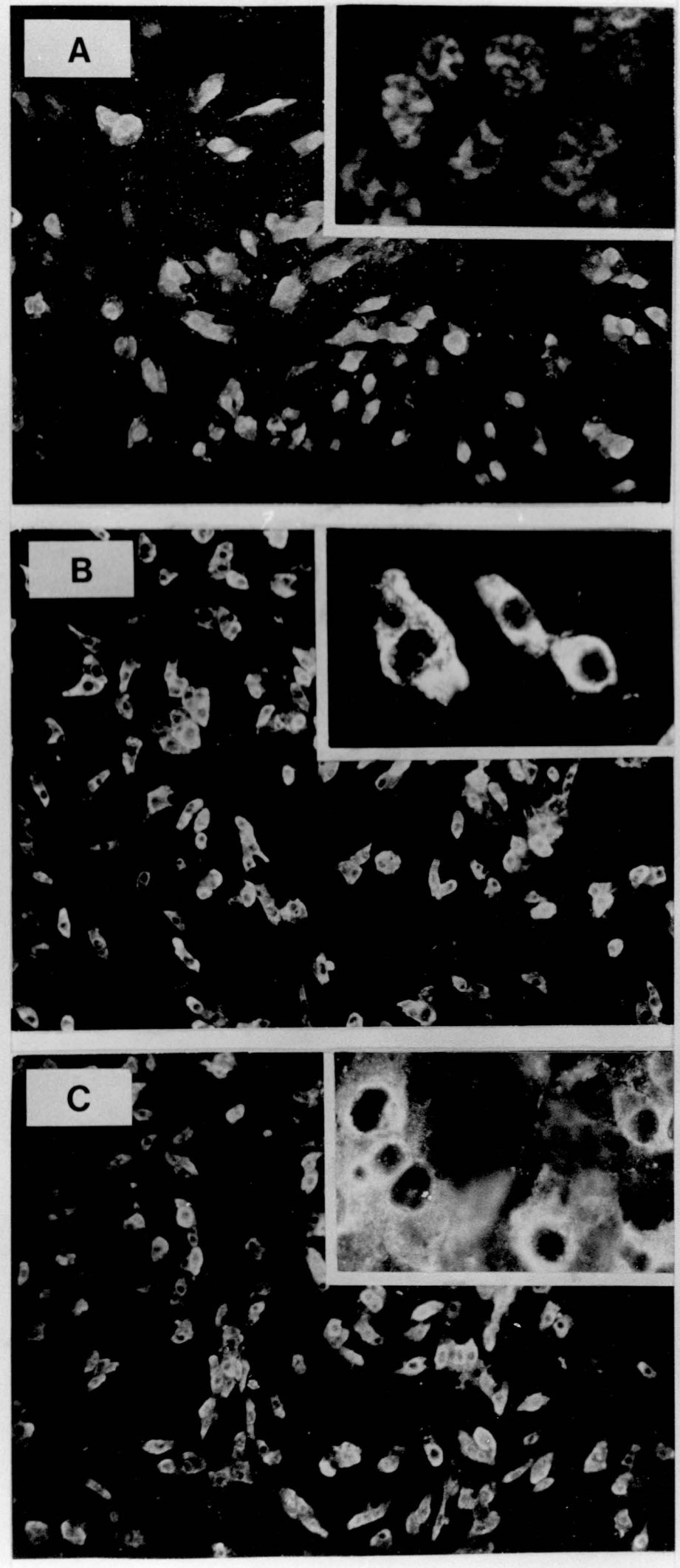


Fig 4

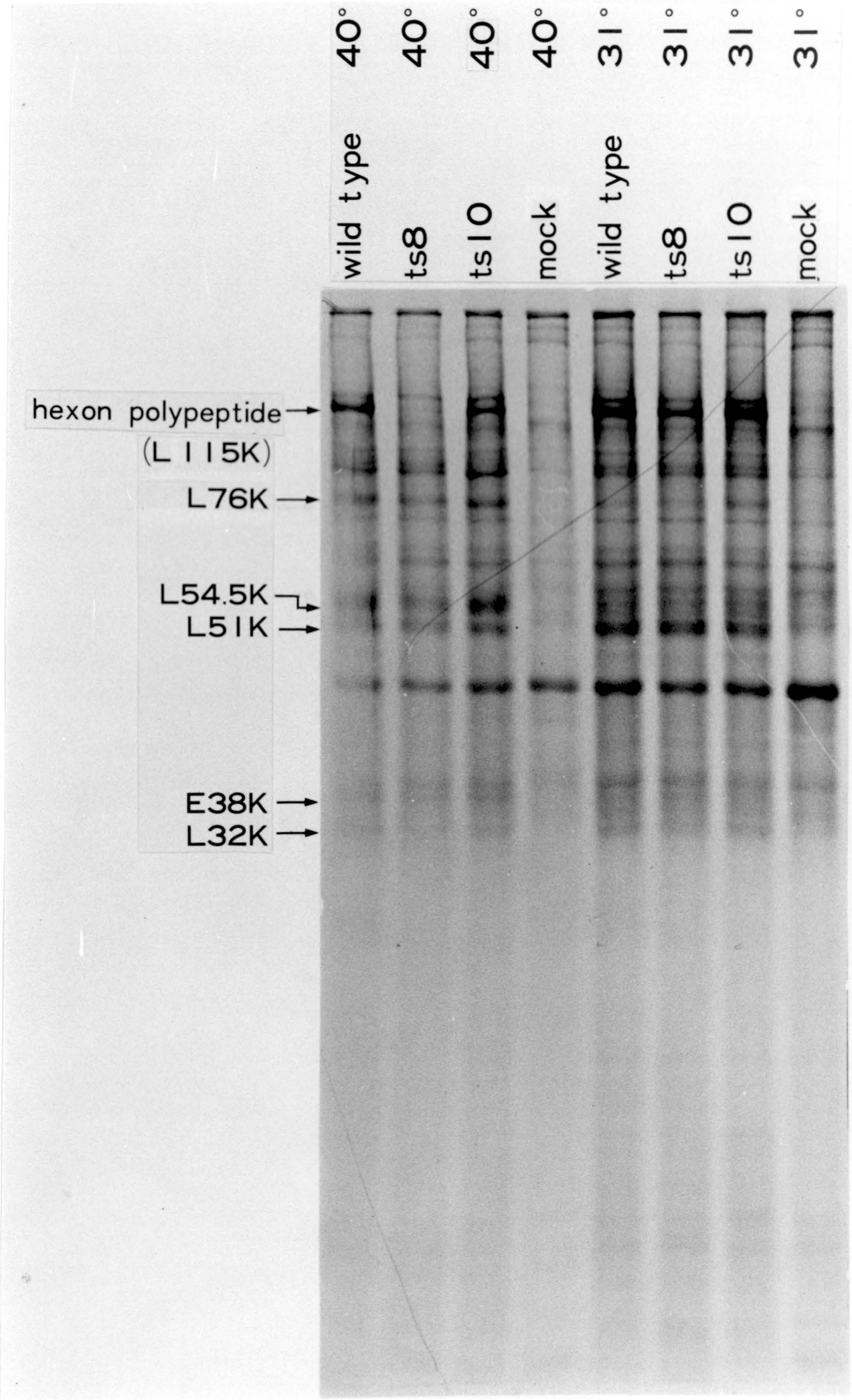

40°
40°
40°
40°
31°
31°
31°
31°
wild type
ts8
ts10
mock
wild type
ts8
ts10
mock
hexon polypeptide
(L 115K)
L76K
L54.5K
L51K
E38K
L32K

Fig 5

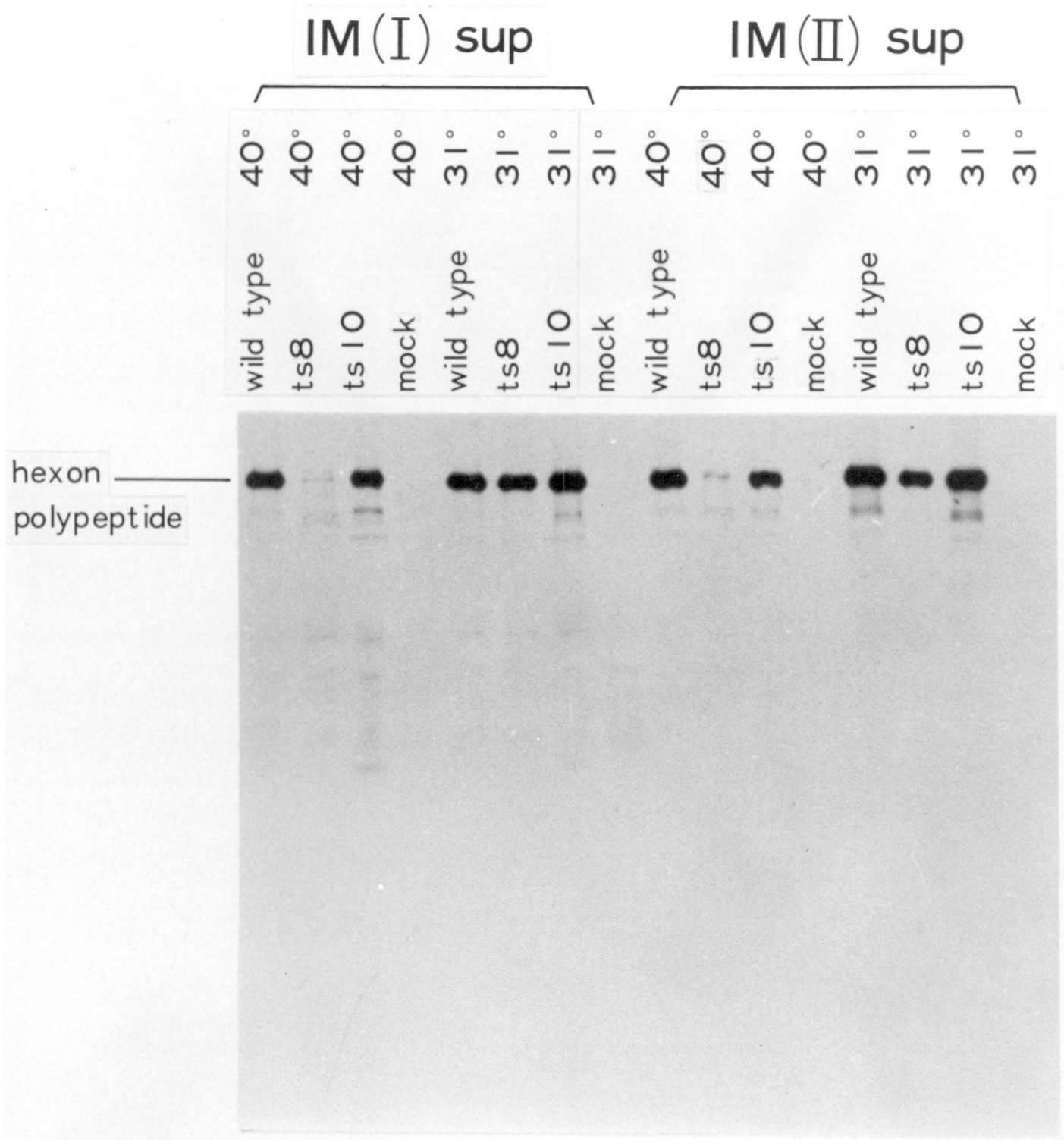

IM(I) sup
IM(II) sup
40° wild type
40° ts8
40° ts10
40° mock
31° wild type
31° ts8
31° ts10
31° mock
40° wild type
40° ts8
40° ts10
40° mock
31° wild type
31° ts8
31° ts10
31° mock
hexon polypeptide

Fig 6

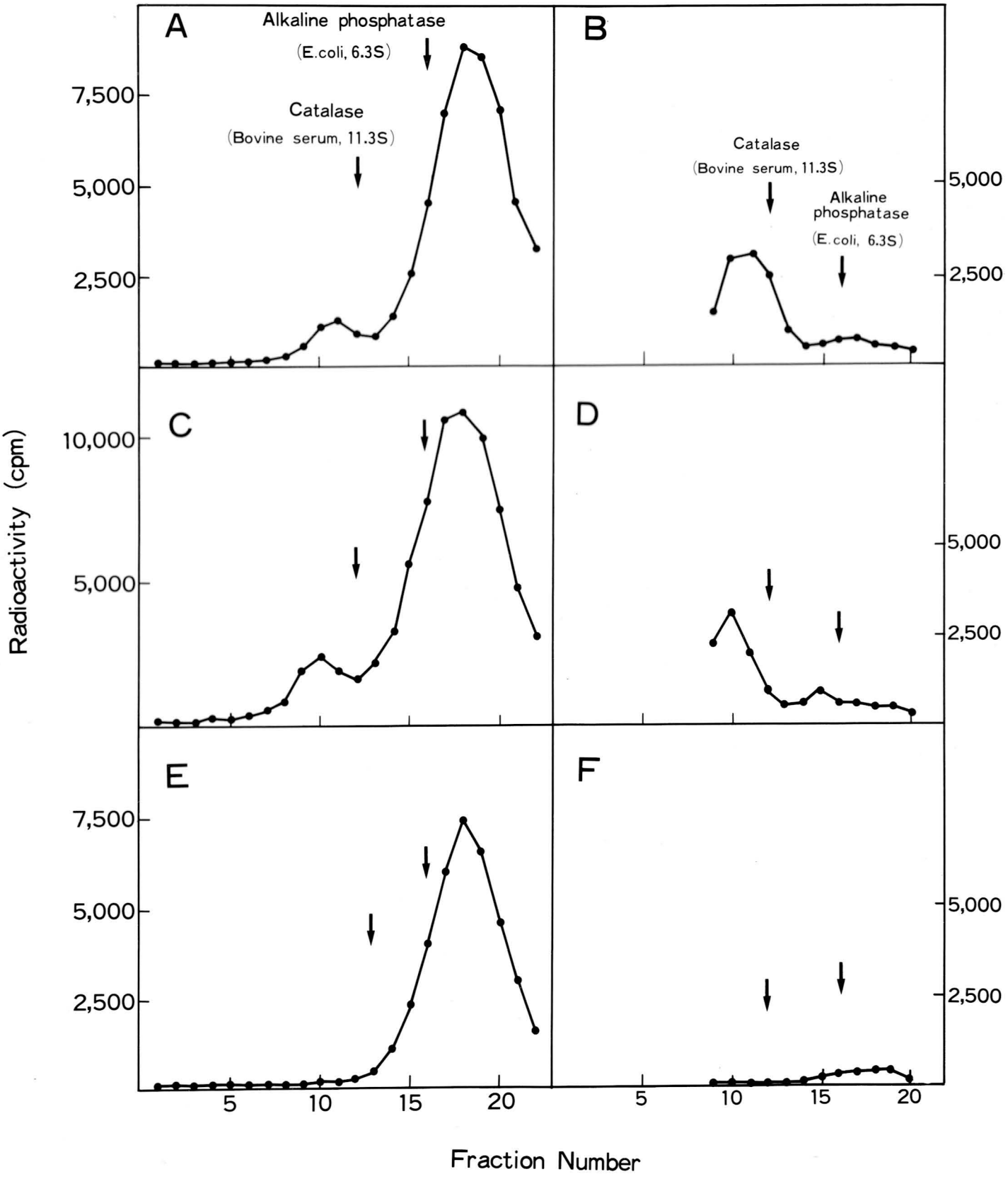
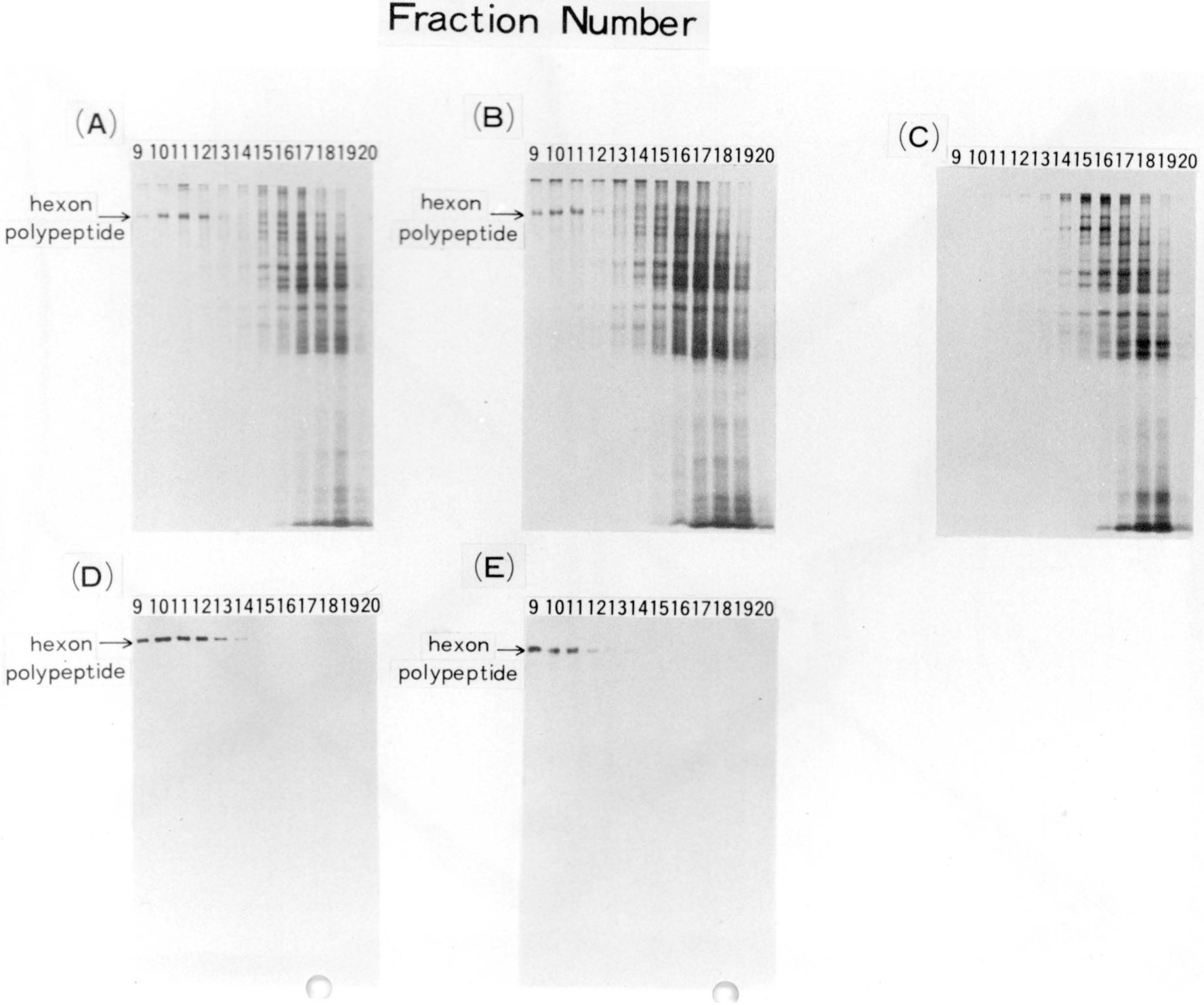

A
Alkaline phosphatase
(E.coli, 6.3S)
Catalase
(Bovine serum, 11.3S)
B
Catalase
(Bovine serum, 11.3S)
Alkaline
phosphatase
(E.coli, 6.3S)
C
D
E
F
7,500
5,000
2,500
10,000
5
10
15
20
Radioactivity (cpm)
Fraction Number

Fig 7.


Fraction Number
(A)
9 10 11 12 13 14 15 16 17 18 19 20
hexon polypeptide
(B)
9 10 11 12 13 14 15 16 17 18 19 20
hexon polypeptide
(C)
9 10 11 12 13 14 15 16 17 18 19 20
(D)
9 10 11 12 13 14 15 16 17 18 19 20
hexon polypeptide
(E)
9 10 11 12 13 14 15 16 17 18 19 20
hexon polypeptide