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NOTES

Late Nonstructural 100,000- and 33,000-Dalton Proteins of Adenovirus Type 2

II. Immunological and Protein Chemical Analysis

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For an immunological analysis of the late adenovirus type 2 nonstructural 100,000-dalton (100K) and 33K proteins, we prepared antisera against sodium dodecyl sulfate-denatured, gel-purified 100K and 33K proteins. These antisera were tested for potential cross-reactivity, since according to a previous report (Axelrod, Virology **87**:366-383, 1978) these two proteins exhibit extensive amino acid homologies. However, immunoprecipitations of 100K and 33K proteins, as well as a sensitive immune replica technique, did not reveal any immunological relationship between these proteins. Therefore, using fingerprint peptide analysis, we investigated the structural relationship between 100K and 33K proteins labeled with a ^{14}C-amino acid mixture or with [^{14}C]proline after digestion with trypsin. We detected only minor, if any, amino acid homologies, indicating that the 100K and 33K proteins are not structurally related.

In adenovirus type 2 (Ad2)-infected cells three virus-encoded late nonstructural proteins having polypeptide molecular weights of about 100,000 (100K) (1-3, 14, 15), 50,000 (50K) (19), and 33,000 (33K) (2, 15, 21) have been characterized in some detail. So far, very little is known about the possible functions of these proteins in the replication cycle of Ad2, but the occurrence of the 100K, 50K, and 33K proteins late in infection suggests that they may be involved in functions necessary for the maturation of virions (e.g., processing of precursor virion proteins, transport, or assembly of virion proteins).

In an attempt to analyze the potential functions of the Ad2 100K and 33K proteins, we decided to determine the exact subcellular locations of these proteins, both by biochemical cell fractionation and by in situ immunofluorescence microscopy in which antisera against the 100K and 33K proteins were used (7). A prerequisite for performing the immunofluorescence experiments was to obtain antisera specific for the 100K and 33K proteins. However, production of such antisera seemed problematic, since several workers have suggested that the 100K and 33K proteins may be related genetically and may share common amino acid sequences. The evidence for this is as follows: (i) the RNAs which program in vitro translation systems to produce the 100K and 33K proteins hybridize to adjoining *Eco*RI fragments (15, 17) and to the same *Bam*HI (17) fragment of Ad2 DNA; (ii) as determined by hybrid-arrested translation, the coding region for the 33K protein on Ad2 DNA seems to be within the region encoding the 100K protein (17); (iii) Axelrod (2) analyzed the tryptic peptides of Ad2 100K and 33K proteins labeled with a ^{14}C-amino acid mixture and found that about two-thirds of the tryptic peptides of the 33K protein were in the 100K protein, implicating a considerable amino acid sequence homology between the two proteins. Therefore, we expected that antiserum against the 33K protein would recognize the 100K protein and vice versa.

To isolate the 100K and 33K proteins, HeLa S_3 cells grown in a suspension culture were infected with Ad2 at a multiplicity of infection of 100. At 36 h postinfection, the cells were lysed in RSB (10 mM Tris-hydrochloride pH 7.2, 10 mM NaCl, 1.5 mM $MgCl_2$) containing 0.5% Nonidet P-40 (Shell Chemicals Co., London, England) and fractionated into a cytoplasmic fraction and a nuclear fraction. The nuclear fraction, which contained most of the 100K protein and all of the 33K protein (7), was separated on preparative sodium dodecyl sulfate (SDS)-poly-

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acrylamide gels. Then the 100K and 33K proteins were eluted electrophoretically from the gel, essentially as described by Lazarides and Weber (13). The extracted proteins were checked for purity by SDS-polyacrylamide gel electrophoresis, using the methods of Laemmli (12) and Maizel (16) (data not shown); these proteins were used to raise antibodies in two guinea pigs (100K protein) and one rabbit (33K protein).

The antisera were first tested by immunoprecipitation of native 100K and 33K proteins that were labeled with $^{32}P_i$ (2, 7) from nuclear extracts of Ad2-infected cells by using the protein A-Sepharose technique (7). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). To our surprise, neither antiserum showed any cross-reaction. The antiserum against the 100K protein quantitatively precipitated the 100K protein without reacting with the 33K protein (Fig. 1, lane c), and the anti-33K serum only recognized the 33K protein (Fig. 1, lane d). A qualitatively similar result was obtained when SDS-denatured 100K and 33K proteins were used as antigens in the immunoprecipitation assay (data not shown). The anti-100K serum also quantitatively removed the 100K protein from cytoplasmic fractions of Ad2-infected cells (data not shown). Control experiments with nonimmune sera and with extracts of uninfected cells confirmed that our antisera reacted only with the Ad2 100K and 33K proteins (data not shown).

To demonstrate further the specificity of our antisera, we characterized them by the sensitive immune replica technique of Saltzgaber-Müller and Schatz (20). HeLa cells were infected with Ad2 and fractionated at 36 h postinfection into a cytoplasmic fraction and a nuclear fraction. The nuclear fraction was electrophoresed on an SDS-polyacrylamide gel and then processed for immune replication in an agarose gel containing antiserum (anti-100K or anti-33K serum). Proteins diffusing from the acrylamide gel into the agarose replica gel formed an immunoprecipitate if they reacted with the embedded antiserum. Any immunoprecipitate in the replica gel then was decorated with ^{125}I-labeled protein A from *Staphylococcus aureus*, which specifically binds to the Fc part of many classes of immunoglobulins (9). Figure 2 shows that the anti-100K serum only reacted with the 100K protein (Fig. 2, lane b) and that the anti-33K serum exclusively recognized the 33K polypeptide (Fig. 2, lane c).

Thus, from the results obtained both by immunoprecipitation and by immune replication we concluded that neither antiserum showed any immunological cross-reaction. This is in accordance with the findings of Oosterom-Dragon and Ginsberg (18), who prepared an antiserum against native Ad5 100K protein that did not react with the Ad5 33K protein. Considering the results of Axelrod (2), this lack of cross-reactivity was difficult to understand. According to the study of Axelrod, the 33K protein has about 12 tryptic peptides in common with the 100K protein and only about 5 peptides of its own. If this extensive amino acid homology exists, the observed failure of the anti-100K serum to recog-

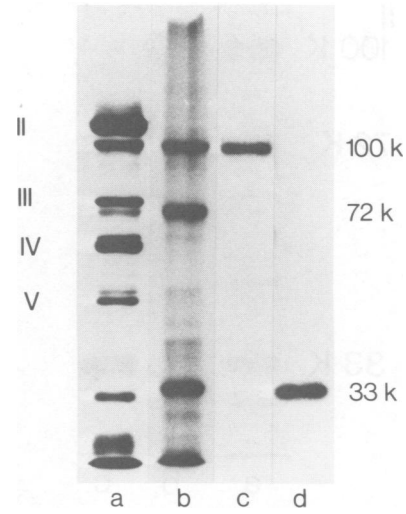

FIG. 1. *Immunoprecipitation of 100K and 33K proteins from nuclear extracts of Ad2-infected HeLa cells by using antisera prepared against 100K and 33K proteins: autoradiograms of $^{32}P_i$-labeled polypeptides. Ad2-infected HeLa S_3 cells were labeled at 36 h postinfection for 2 h with $^{32}P_i$ (200 μCi/ml; 285 Ci/mg of P) in growth medium containing only 1/10 the normal phosphate concentration. The cells were lysed in RSB containing 1 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40 and then fractionated into a cytoplasmic fraction and a nuclear fraction by centrifugation at 10,000 × g for 10 min. Nuclear fractions were sonically treated in Tris buffer (pH 7.4)-0.5 M NaCl-1% Nonidet P-40-1 mM phenylmethylsulfonyl fluoride for 30 s on ice, and the lysate was centrifuged at 15,000 × g for 30 min at 4°C. The supernatant (nuclear extract) was diluted with the same volume of RSB and used for immunoprecipitations. The immunoprecipitates were analyzed on a 7.5% SDS-polyacrylamide gel. Lane a, Homogenate of Ad2-infected cells labeled with ^{14}C-amino acids (protein marker); lane b, $^{32}P_i$-labeled nuclear extract (original reaction mixture); lane c, immunoprecipitate with anti-100K serum; lane d, immunoprecipitate with anti-33 K serum. Control experiments with nonimmune sera and with extracts of uninfected cells confirmed that our antisera reacted only with the Ad2 100K and 33K proteins (data not shown).*

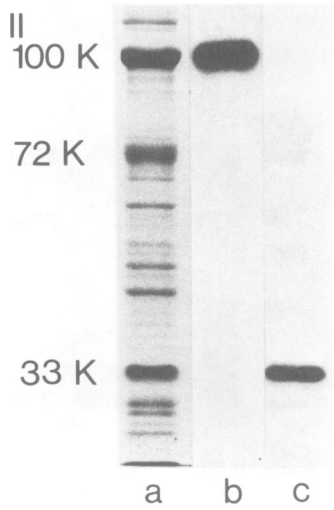

FIG. 2. *Immune replicas showing the reaction between Ad2-specific proteins separated by SDS-polyacrylamide gel electrophoresis and antiserum against 100K or 33K protein. Nuclear fractions were prepared from Ad2-infected HeLa S_3 cells at 36 h postinfection as described in the legend to Fig. 1, separated on a 7.5% SDS-polyacrylamide gel, and subjected to immune replication (20). The acrylamide gel was placed for 20 h onto an agarose layer (1% agarose in 0.14 M NaCl-10 mM sodium phospate [pH 7.4]-0.4% Triton X-100) containing anti-100K or anti-33K serum in order to allow diffusion of proteins from the acrylamide gel into the agarose gel. Then the acrylamide gel was removed, and the agarose layer was washed for 48 h with isotonic saline. To detect immunocomplexes formed in the agarose gel, the gel was incubated in isotonic saline containing ^{125}I-labeled S. aureus protein A iodinated by the method of Greenwood et al. (10). The agarose gel was washed extensively in isotonic saline, dried, and autoradiographed.*

nize the 33K protein and vice versa could be explained only if the amino acid sequences common to both proteins were not immunogenic or were only weakly immunogenic, or, alternatively, if the common sequences in both polypeptides formed an inner core which was not available to the outside environment even after SDS denaturation.

Therefore, we investigated the suggested amino acid sequence homology between the 33K and 100K proteins by using two-dimensional peptide mapping. In our first experiment we analyzed the tryptic peptides of the 100K and 33K proteins labeled with a ^{14}C-amino acid mixture by using the same protocol as Axelrod (2). Figure 3 shows the tryptic peptide patterns of the 100K protein (Fig. 3A), the 33K protein (Fig. 3B), and a mixture of the two (Fig. 3C). The 100K protein produced about 100 discrete peptide spots, and the 33K protein produced about 30 spots. It is important that only very little radioactive material remained at the origin (Fig. 3, arrowheads), indicating complete digestion of both proteins by trypsin, as well as a good separation of all peptides. A thorough analysis of the peptide maps shown in Fig. 3 revealed a maximum of three peptides (peptides x, y, and z) common to both the 100K protein and the 33K protein. However, since these peptides only formed minor spots (i.e., presumably were small peptides), it was difficult to identify them definitely, especially in the mixture.

Reading and interpretation of peptide maps become difficult when a large number of spots have to be analyzed (Fig. 3). To reduce the number of labeled peptides in the 100K and 33K maps, thereby facilitating the identification of possible common peptides, we analyzed the tryptic peptides of [^{14}C]proline-labeled 100K and 33K proteins. Proteins were labeled with proline because proline interrupts an α-helical arrangement of amino acids in proteins and, therefore, presumably is located in structurally important areas of proteins. Thus, an analysis of [^{14}C]proline-labeled tryptic peptides of the 100K and 33K proteins should have allowed us to identify homologous areas in the amino acid sequences of these proteins. Figure 4 shows the tryptic peptide maps of [^{14}C]proline-labeled 100K (Fig. 4A) and 33K (Fig. 4B) proteins. For the 100K protein (Fig. 4A), about 35 peptides could be identified, and about 12 could be identified for the 33K protein (Fig. 4B). A comparison of a mixture of 100K and 33K peptides (Fig. 4C) showed that the two proteins did not have a single proline peptide in common. Therefore, we concluded that there are at least no major amino acid homologies between the two proteins.

This conclusion was further confirmed by a fingerprint analysis of the chymotryptic peptides of 100K and 33K proteins labeled with a ^{14}C-amino acid mixture. Only about 5 peptides of about 125 resolved peptides for the 100K protein and about 45 peptides for the 33K protein had the same mobility in both 100K and 33K peptide maps (data not shown). The low number of common peptides observed after a fingerprint analysis of 100K and 33K proteins labeled with a ^{14}C-amino acid mixture does not argue against our conclusion. Considering the size of these proteins, a low number of common peptides might be expected for statistical reasons. In addition, our immunological data strongly support our finding that the Ad2 100K and 33K proteins are not structurally related.

To explain the discrepancy between the data

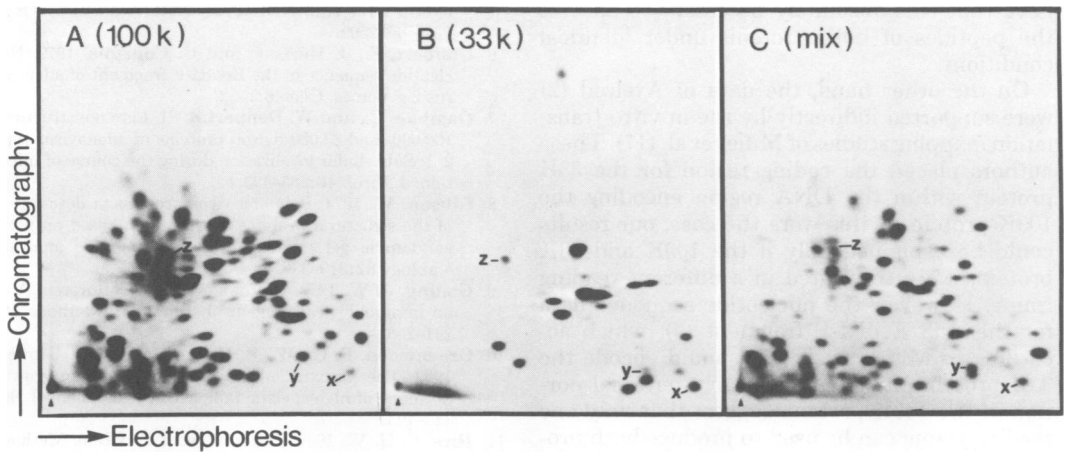

FIG. 3. *Tryptic peptide maps of Ad2 100K and 33K proteins labeled with a ^{14}C-amino acid mixture. Ad2-infected HeLa S_3 cells were labeled at 26 h postinfection for 3 h with a ^{14}C-amino acid mixture (50 μCi/ml) in growth medium containing only 1/10 the normal amino acid concentration. The cells were subsequently fractionated as described in the legend to Fig. 1. The 100K and 33K proteins were isolated from preparative SDS-polyacrylamide gels on which the nuclear fraction or immunoprecipitates of nuclear extracts with anti-100K or anti-33K had been separated (5). Samples of the extracted proteins were checked for purity by SDS-polyacrylamide gel electrophoresis (data not shown). The rest of each preparation was oxidized with performic acid as described by Hirs (11). The oxidized proteins were digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin and subjected to peptide analysis on thin-layer cellulose plates (8), using the modification described by Deppert and Walter (5). Peptides were visualized by autoradiography.*

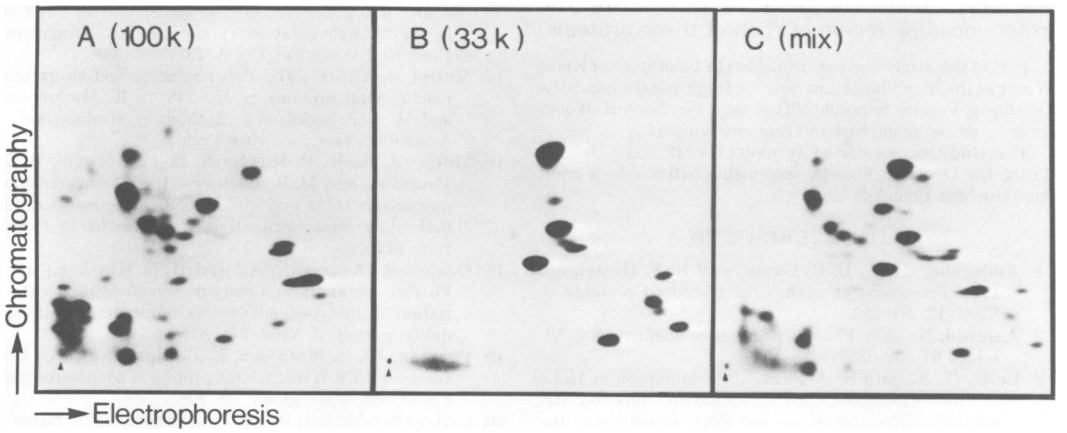

FIG. 4. *Tryptic peptide maps of Ad2 100K and 33K proteins labeled with [^{14}C]proline. Ad2-infected HeLa S_3 cells were labeled at 26 h postinfection for 3 h with [^{14}C]proline (40 μCi/ml; 282 mCi/mmol) in normal growth medium and then fractionated. The 100K and 33K proteins were isolated and processed for peptide analysis as described in the legend to Fig. 3. Peptides were visualized by fluorography after the thin-layer plates were immersed in a solution of 0.4% (wt/vol) PPO(2,5-diphenyloxazole) in 2-methylnaphthalene (4).*

of Axelrod (2) and our data, the following points concerning the study of Axelrod should be considered critically: (i) it seems that relatively large amounts of the 100K and 33K proteins were not digested by trypsin, leading to a large smear in the area of the origin, and most probably this inefficient cleavage was responsible for the low number of discrete peptide spots (roughly one-half the number in our experiments) on the peptide maps of Axelrod; and (ii) Axelrod did not analyze a mixture of the 100K and 33K tryptic peptides on the same plate, but only compared the two individual maps of the 100K and 33K proteins visually or by superimposing. However, for a thorough comparison of the peptide patterns of two individual proteins, we be-

lieve that it is absolutely necessary to analyze the peptides of both proteins under identical conditions.

On the other hand, the data of Axelrod (2) were supported indirectly by the in vitro translation mapping studies of Miller et al. (17). These authors placed the coding region for the 33K protein within the DNA region encoding the 100K protein. If this were the case, our results could be explained only if the 100K and 33K proteins were translated in a different reading frame. However, the nucleotide sequence data for the Ad2 *Eco*RI-F fragment (6), which according to Miller et al. (17) should encode the 33K protein plus a large carboxy-terminal portion of the 100K protein, suggest that only one reading frame can be used to produce both proteins. Therefore, our results favor the assumption that the 100K and 33K proteins are encoded by separate regions on the Ad2 DNA. This was also suggested by the S1 nuclease mapping data of Berget (17), which placed the coding region for the 33K protein slightly farther to the right on the standard map of Ad2 DNA than the data of Miller et al. (17). However, ultimate resolution of this conflict should come with elucidation of the amino acid sequences of the 100K and 33K proteins, which will allow us to describe the exact encoding region of each of these proteins.

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LITERATURE CITED

1. **Anderson, C. W., P. R. Baum, and R. F. Gesteland.** 1973. Processing of adenovirus 2-induced proteins. J. Virol. **12**:241-252.
2. **Axelrod, N.** 1978. Phosphoproteins of adenovirus 2. Virology **87**:366-383.
3. **Beltz, G. A., and S. J. Flint.** 1979. Inhibition of HeLa cell protein synthesis during adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. J. Mol. Biol. **131**:353-373.
4. **Bonner, W. M., and J. D. Stedman.** 1978. Efficient fluorography of ^{3}H and ^{14}C on thin layers. Anal. Biochem. **89**:247-256.
5. **Deppert, W., and G. Walter.** 1976. Simian virus 40 (SV40) tumor-specific proteins in nucleus and plasma membrane of HeLa cells infected by adenovirus 2-SV40 hybrid virus Ad2^{+}ND2. Proc. Natl. Acad. Sci. U.S.A. **73**:2505-2509.
6. **Galibert, F., J. Hérissé, and G. Courtois.** 1979. Nucleotide sequence of the EcoRI-F fragment of adenovirus 2 genomes. Gene **6**:1-22.
7. **Gambke, C., and W. Deppert.** 1981. Late nonstructural 100,000- and 33,000-dalton proteins of adenovirus type 2. I. Subcellular localization during the course of infection. J. Virol. **40**:585-593.
8. **Gibson, W.** 1974. Polyoma virus proteins: a description of the structural proteins of the virion based on polyacrylamide gel electrophoresis and peptide analysis. Virology **62**:319-336.
9. **Goding, J. W.** 1978. Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods **20**: 241-253.
10. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**: 114-123.
11. **Hirs, C. H. W.** 1967. Performic acid oxidation. Methods Enzymol. **11**:198-199.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
13. **Lazarides, E., and K. Weber.** 1974. Actin antibody: the specific visualization of actin filaments in non-muscle cells. Proc. Natl. Acad. Sci. U.S.A. **71**:2268-2272.
14. **Lewis, J. B., C. W. Anderson, and J. F. Atkins.** 1977. Further mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. Cell **12**:37-44.
15. **Lewis, J. B., J. F. Atkins, C. W. Anderson, P. R. Baum, and R. F. Gesteland.** 1975. Mapping of late adenovirus genes by cell-free translation of RNA selected by hybridization to specific DNA fragments. Proc. Natl. Acad. Sci. U.S.A. **72**:1344-1348.
16. **Maizel, J. V., Jr.** 1971. Polyacrylamide gel electrophoresis of viral proteins, p. 179-246. *In* K. Maramorosh and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press, Inc., New York.
17. **Miller, J. S., R. P. Ricciardi, B. E. Roberts, B. M. Paterson, and M. B. Mathews.** 1980. Arrangement of messenger RNAs and protein coding sequences in the major late transcription unit of adenovirus 2. J. Mol. Biol. **142**:455-488.
18. **Oosterom-Dragon, E. A., and H. S. Ginsberg.** 1980. Purification and preliminary immunological characterization of the type 5 adenovirus, nonstructural 100,000-dalton protein. J. Virol. **33**:1203-1207.
19. **Persson, H., B. Mathisen, L. Philipson, and U. Pettersson.** 1979. A maturation protein in adenovirus morphogenesis. Virology **93**:198-208.
20. **Saltzgaber-Müller, J., and G. Schatz.** 1978. Heme is necessary for the accumulation and assembly of cytochrome c oxidase subunits in *Saccharomyces cerevisiae*. J. Biol. Chem. **253**:305-310.
21. **Walter, G., and H. Martin.** 1975. Simian virus 40-specific proteins in HeLa cells infected with nondefective adenovirus 2-simian virus 40 hybrid viruses. J. Virol. **16**: 1236-1247.