DNA Binding Proteins in the Cytoplasm and in a Nuclear Membrane Complex Isolated from Uninfected and Adenovirus 2 Infected Cells†

G. Shanmugam,* Saumya Bhaduri, Max Arens, and Maurice Green

ABSTRACT: The DNA binding proteins in a nuclear membrane fraction that can synthesize DNA *in vitro* (referred to as "nuclear membrane complex") and in the cytoplasm of adenovirus infected and uninfected cells were isolated and characterized. Suspension cultures of human KB cells infected with human adenovirus 2 were treated with 25 μg/ml of arabinosylcytosine starting at 2 hr to block the synthesis of viral structural proteins, and then labeled with [^{3}H]leucine from 6 to 24 hr after infection. Uninfected cells were treated similarly and labeled with [^{14}C]leucine. The ^{3}H-labeled proteins (infected cells) and ^{14}C-labeled proteins (uninfected cells) isolated from the cytoplasm were mixed, as were the corresponding proteins isolated from the membrane complex, and each mixture was fractionated by stepwise elution from single-stranded DNA-cellulose columns. From 50 to 60% of the labeled protein in the membrane complex from infected cells and 40 to 50% of that from uninfected cells bound to DNA-cellulose in 0.05 M NaCl. Much less protein from the cytoplasm was bound to DNA cellulose, 20% from infected cells and 11% from uninfected cells. Gel electrophoresis of the mixture of ^{3}H- and ^{14}C-labeled proteins eluted from DNA-cellulose by different concentrations of NaCl revealed the following. (1) The 0.15 and 0.40 M NaCl eluates from the membrane complex of infected and uninfected cells contained a heterogeneous mixture of similar polypeptides. (2) The 0.6 M NaCl eluate from the membrane complex derived from infected cells contained two major DNA binding proteins with molecular weights of 75,000 and 45,000 that were absent from uninfected cells. Large quantities of these two proteins were present in highly purified form in the 0.6 M NaCl eluate from the cytoplasm of infected cells. The DNA binding proteins of molecular weight 75,000 and 45,000 that are present in the cytoplasm are identical with those present in the membrane complex, as established by coelectrophoresis. (3) Two major cell-specific proteins of molecular weight 40,000 and 15,000–17,000 were present in the 2 M NaCl eluate of the membrane complex from uninfected and infected cells. A major cell-specific protein of molecular weight 33,000 was present in the 0.15 and 0.4 M NaCl eluates of the uninfected and infected cell cytoplasmic fractions. Analysis of cells labeled at 2–6 hr after infection in the absence of arabinosyl cytosine indicated that the synthesis of the DNA binding proteins of molecular weight 75,000 and 45,000 begins early after infection prior to the onset of viral DNA replication.

The mechanism of DNA replication in bacterial systems is surprisingly complex, involving the interaction of multiple protein and enzyme components. DNA replication in eukaryotic cells may be even more intricate in view of their much larger size and genetic complexity. The replication of viral DNA in cultured mammalian cells provides an excellent model to study the mechanism of DNA synthesis in eukaryotic cells. An especially attractive and well-studied system for investigating the synthesis of linear duplex DNA molecules is human KB cells infected with human adenovirus 2 (Green, 1970; Green *et al.*, 1970). Late after infection at 18 hr, the replication of adenovirus DNA of molecular weight 23 × 10^6 can be studied in the absence of host cell DNA synthesis (Piña and Green, 1969).

We recently reported that newly synthesized adenovirus 2 DNA can be isolated in association with preparations of nuclear membrane fractions containing two new proteins of molecular weight 75,000 and 45,000 that are not detected in uninfected cells (Yamashita and Green, 1974). The nuclear membrane fraction isolated by centrifugation on a discontinuous sucrose gradient (referred to as "nuclear membrane complex") possesses both DNA polymerase activity and a virus-specific endonuclease and can synthesize adenovirus DNA sequences *in vitro* (Yamashita *et al.*, 1975). The association of the 75,000 and 45,000 molecular weight proteins with the nuclear membrane complex suggests that they may function in DNA synthesis. This possibility is further strengthened by the isolation of two DNA binding proteins of similar molecular weights, 72,000 and 48,000, from monkey kidney cells abortively infected with adenovirus 5 (Van der Vliet and Levine, 1973). This finding and the fact that DNA binding proteins appear to play a role in the replication of bacteriophage T4 DNA (Alberts and Frey, 1970; Alberts, 1970; Delius *et al.*, 1972) prompted us to carry out a systematic analysis of the DNA binding proteins present in the nuclear membrane complex and in the cytoplasm of adenovirus 2 infected cells. For comparison, uninfected KB cells were analyzed simultaneously by double labeling techniques to detect cell-specific DNA binding proteins. We show here that the proteins of molecular weight 75,000 and 45,000 isolated from the membrane complex bind strongly to single-stranded DNA cellulose columns, are synthesized early after infection, and are not detected in uninfected cells. These DNA binding proteins can be isolated in large quantities from the cytoplasm in highly purified form by

† From the Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Missouri 63110. *Received July 29, 1974.* This work was supported by funds from Public Health Service Grant AI-01725 from the National Institute of Allergy and Infectious Diseases and from Contract NO1 CP 43359 from the Virus Cancer Program of the National Cancer Institute. M.G. is a Research Career Awardee of the National Institutes of Health (5K6-AI-4739). Paper III in a series entitled Adenovirus DNA Replication. Paper I is Yamashita and Green (1974). Paper II is Yamashita *et al.* (1975).

chromatography on DNA-cellulose columns. The presence of several major DNA binding proteins in uninfected cells is also documented.

Materials and Methods

Materials. L-[4,5-N-^{3}H]Leucine (30–50 Ci/mmol), L-[U-^{14}C]leucine (270 Ci/mol), L-[^{3}H]valine (2–10 Ci/mmol), L-[^{3}H]threonine (2–10 Ci/mmol), and Aquasol scintillation fluid were purchased from New England Nuclear; DNase I (EC 3.1.4.5) from Worthington Biochemical Corp.; calf thymus DNA (Type I) and sodium dodecyl sulfate from Sigma Chem. Co.; Whatman cellulose powder, standard grade from W & R Balston Ltd; and acrylamide and bisacrylamide from Bio-Rad Laboratories. All other materials were of analytical grade.

Cell Culture, Virus, and Labeling of Infected and Uninfected Cells. A clonal KB cell line was grown in suspension in Eagle's minimal essential medium containing 5% horse serum (MEM).[1] For infection, cells were centrifuged and resuspended at 1×10^7 cells/ml in MEM without serum, and adenovirus 2 (strain 38-2, plaque 4, free of adenovirus-associated viruses) was added at an input multiplicity of 100 PFU/cell (Piña and Green, 1969). After 1 hr for adsorption at 37°, the cell suspension was diluted with complete medium to yield a cell density of 3×10^5 cells/ml, and further incubated in suspension at 37°. At 2 hr after infection, 25 μg/ml of Ara C was added. At 6 hr cells were centrifuged, washed with leucine-free MEM at 37°, and resuspended in leucine-free MEM. Ara C (25 μg/ml) and [^{3}H]leucine (2 μCi/ml) were added and the cells were incubated until 24 hr after infection. Uninfected cells were labeled with [^{14}C]leucine (0.17 μCi/ml) in the presence of Ara C and treated as described above for infected cells except that no virus was added.

For the preparation of the nuclear membrane complex and the cytoplasmic fraction early after infection, the cells were concentrated and infected as described above. At 2 hr after infection, cells were centrifuged, washed with leucine-free MEM at 37°, and resuspended in leucine-free MEM to which [^{3}H]leucine (4 μCi/ml) was added. Uninfected cells were treated similarly and labeled with [^{14}C]leucine (0.34 μCi/ml). Cell cultures were harvested at 6 hr after infection and processed as described below.

Preparation of the Cytoplasmic Fraction and the "Nuclear Membrane Complex." Adenovirus 2 infected and uninfected KB cells were harvested at 24 hr after infection, washed with phosphate-buffered saline (PBS) lacking Mg^{2+} and Ca^{2+} (Dulbecco and Vogt, 1954), resuspended at 10^6–10^7 cells/ml in sterile RSB (reticulocyte standard buffer: 0.01 M Tris-HCl (pH 7.4)–0.01 M NaCl–0.0015 M $MgCl^2$) and placed in an ice bath. After 15 min, cells were disrupted with 15–20 strokes of a tight-fitting Dounce homogenizer (Kontes Glass Co.) and centrifuged through 5 ml of a 25% sucrose cushion in RSB at 2000*g* for 10 min at 4° to separate the supernatant cytoplasmic fraction and the nuclear pellet. Nuclei were washed with cold RSB, centrifuged through 5 ml of 25% sucrose in RSB, and resuspended at 10^6–10^7 nuclei/ml in TKM buffer (0.05 M Tris-HCl (pH 7.5)–0.025 M KCl–0.005 M $MgCl_2$–0.003 M dithiothreitol). The membrane complex was isolated from purified nuclei by the discontinuous sucrose density gradient procedure (Kashnig and Kasper, 1969; Yamashita and Green, 1974), as described below. The suspension of nuclei was sonically disrupted at full power in a Fischer sonic oscillator at 0° to break over 98% of the nuclei (usually 45 sec), as monitored by phase microscopy. Sodium citrate was added to 10% and the suspension was stirred in ice for 15 min; 20 ml were layered above 5-ml layers of 1.22 and 1.20 g/ml of sucrose in TKM buffer containing 10% sodium citrate, and centrifuged in a Spinco SW 27 rotor at 25,000 rpm for 60 min at 4°. The visible band of the membrane complex above the 1.20 g/ml of sucrose cushion representing the inner nuclear membrane (Kashnig and Kasper, 1969) was collected.

For cochromatography of the DNA binding proteins, preparations of [^{3}H]leucine- and [^{14}C]leucine-labeled membrane complex from adenovirus 2 infected and uninfected cells, respectively, were mixed and incubated at 22° for 30 min with 20 μg/ml of DNase I after the addition of 50 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. The mixture was dialyzed at 4° against two changes of 4 l. of dialysis buffer (20 mM Tris-HCl (pH 8.1)–5 mM EDTA–1 mM 2-mercaptoethanol–0.05 M NaCl–10% glycerol). Mixtures of ^{3}H- and ^{14}C-labeled cytoplasmic fractions isolated from infected and uninfected cells were prepared in the same manner.

DNA-Cellulose Chromatography. Single-stranded DNA-cellulose was prepared essentially as described by Litman (1968). Calf thymus DNA (2 mg/ml in 1 mM NaCl) was denatured by heating at 100° for 10 min and rapidly cooled. Denatured DNA (40 ml) was added to 5 g of acid-washed cellulose powder, dried in air overnight in an 800-ml glass beaker, and scraped into 100 ml of absolute ethanol. The suspension was irradiated with an ultraviolet lamp (Mineralight, Ultra Violet Products, Inc.), placed 12 cm above the surface of the suspension for 25 min with slow stirring. The DNA-cellulose was collected on Whatman No. 2 paper, washed three times with 300 ml of 1 mM NaCl by vacuum filtration, and dried in air. Under these conditions, 80% of the DNA was bound to the cellulose. To prepare a 0.8×4 cm column, 0.6 g of DNA-cellulose was suspended in 10 ml of column buffer (20 mM Tris-HCl (pH 8.1)–5 mM EDTA–1 mM 2-mercaptoethanol) containing 0.05 M NaCl. The column was washed and eluted at a flow rate of 6 ml/hr and 0.4-ml fractions were collected.

Electrophoresis on Sodium Dodecyl Sulfate–Polyacrylamide Gels. Following DNA-cellulose column chromatography, the desired fractions were pooled and precipitated with 20% Cl_3CCOOH in the presence of 200 μg of bovine serum albumin. The precipitates were washed with 0.5% Cl_3CCOOH and suspended in 0.01 M sodium phosphate buffer (pH 7.0). After addition of sodium dodecyl sulfate (1%), 2-mercaptoethanol (1%), sucrose (10%), and Bromophenol Blue (0.003%), the samples were heated for 2 min at 100°. Electrophoresis was performed at 10 mA/gel for 3–4 hr in 0.6×10 cm tubes containing 6% polyacrylamide and 0.16% bisacrylamide according to Maizel (1969). The gels were fractionated (1 mm/fraction) using a Gilson gel fractionator. The following molecular weight markers for polypeptides were used: [^{3}H]valine- and [^{3}H]threonine-labeled adenovirus 2 hexon and penton polypeptides purified by DEAE-cellulose chromatography and assumed to possess molecular weights of 120,000 and 70,000, respectively (Maizel *et al.*, 1968); [^{3}H]leucine-labeled polypeptide of molec-

[1] Abbreviations used are: Ara C, 1-β-D-arabinosylcytosine; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline; TKM buffer, 0.05 M Tris-HCl (pH 7.5)–0.025 M KCl–0.005 M $MgCl_2$–0.003 M dithiothreitol; RSB, reticulocyte standard buffer, 0.01 M Tris-HCl (pH 7.4)–0.01 M NaCl–0.0015 M $MgCl_2$.

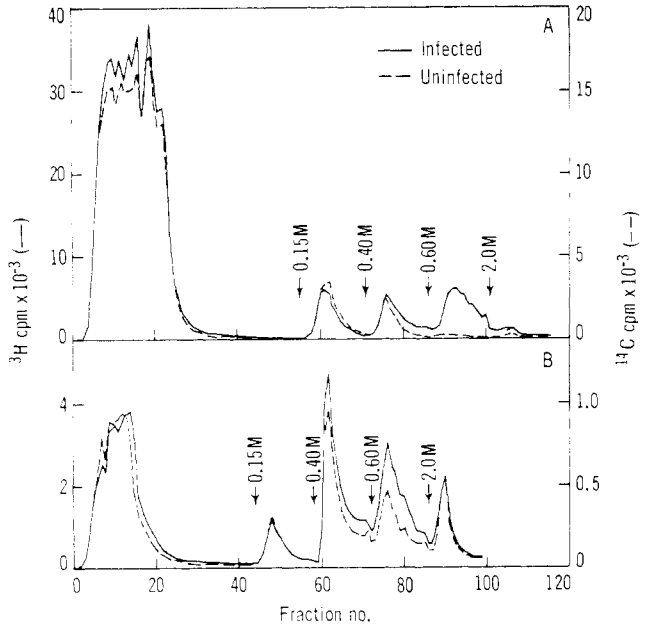

FIGURE 1: DNA-cellulose column chromatography of DNA binding proteins present in the cytoplasm (A) and membrane complex (B) of adenovirus 2 infected and uninfected KB cells. [^{3}H]Leucine-labeled cytoplasm from infected cells and [^{14}C]leucine-labeled cytoplasm from uninfected cells were mixed, treated with DNase, and dialyzed as described under Materials and Methods. [^{3}H]Leucine-labeled membrane complex from infected cells and [^{14}C]leucine-labeled membrane complex from uninfected cells were mixed and treated in the same manner: 6 ml of the mixed cytoplasm containing 20.3 × 10^6 ^{3}H cpm and 10.4 × 10^6 ^{14}C cpm, or 4.6 ml of mixed membrane complex containing 10.1 × 10^6 ^{3}H cpm and 2.16 × 10^6 ^{14}C cpm were loaded onto 0.8 × 4 cm columns of DNA-cellulose and washed with 12 ml of column buffer (20 mM Tris-HCl (pH 8.1), 5 mM EDTA, and 1 mM 2-mercaptoethanol) containing 0.05 M NaCl. The DNA binding proteins were eluted stepwise with 5 ml each of 0.15, 0.4, 0.6, and 2 M NaCl in column buffer.

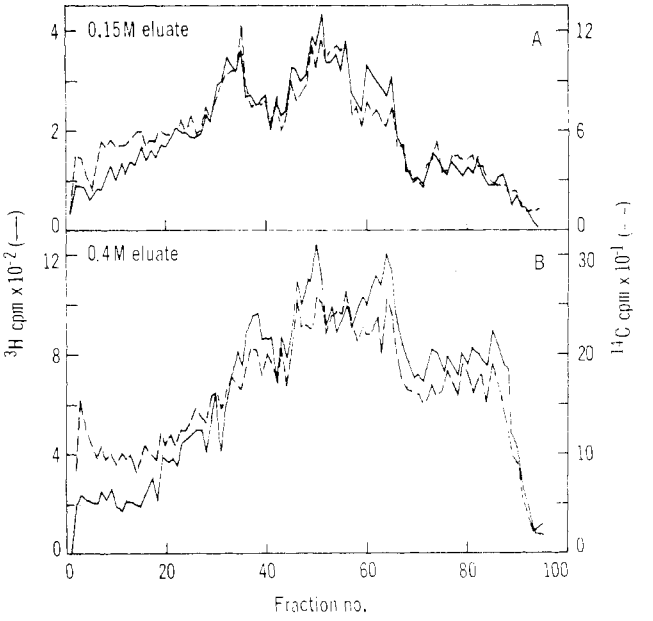

FIGURE 2: Coelectrophoresis on a sodium dodecyl sulfate–polyacrylamide gel of proteins from the membrane complex from infected ^{3}H and uninfected ^{14}C cells eluted from DNA-cellulose with 0.15 M NaCl (A) and 0.4 M NaCl (B). The NaCl eluates of Figure 1B, fractions 46–52 of the 0.15 M NaCl eluate, or fractions 60–66 of the 0.4 M NaCl eluate, were pooled and precipitated with Cl_3CCOOH. The precipitate derived from the 0.15 M NaCl eluate (55,400 ^{3}H cpm and 13,200 ^{14}C cpm) and that from the 0.4 M NaCl eluate (237,300 ^{3}H cpm and 42,900 ^{14}C cpm) were electrophoresed on 6% polyacrylamide gels as described under Materials and Methods.

ular weight 31,000 (P31) and 18,000 (P18) from the murine sarcoma-leukemia virus (Moloney strain) (Shanmugam *et al.*, 1972). The marker proteins were electrophoresed on parallel gels and molecular weights were calculated according to Weber and Osborn (1969).

Measurement of Radioactivity. Fractions (10 μl) from DNA-cellulose columns or 1-mm gel fractions in 0.3 ml of H_2O were counted in 10 ml of Aquasol in a Beckman liquid scintillation counter. In double label experiments, the spillover of ^{14}C into the ^{3}H channel (12–14%) was calculated with appropriate standards and the ^{3}H radioactivity corrected accordingly.

Results

DNA-Cellulose Chromatography of DNA Binding Proteins. To isolate and characterize virus-specific proteins that bind to DNA, adenovirus-infected cells were labeled with [^{3}H]leucine from 6 to 24 hr after infection. Ara C was added at 2 hr to prevent the synthesis of viral DNA and late viral proteins. Uninfected cells were labeled with [^{14}C]leucine under the same conditions. The cytoplasmic fraction and the nuclear membrane complex were isolated from infected and uninfected cells and fractionated on single-stranded DNA-cellulose. Figure 1A shows the elution profile on DNA-cellulose after cochromatography of a mixture of [^{3}H]leucine- and [^{14}C]leucine-labeled proteins from the cytoplasm of infected and uninfected cells, respectively. Figure 1B shows the corresponding elution profile after cochromatography of proteins isolated from the nuclear membrane complex. About 20% of the ^{3}H-labeled proteins in the cytoplasm of infected cells and 11% of the ^{14}C-labeled proteins in the cytoplasm of uninfected cells bound to DNA-cellulose in 0.05 M NaCl (Figure 1A). The membrane complex contained a much higher concentration of DNA binding proteins, from 50 to 60% and 40 to 50% of the labeled proteins from infected and uninfected cells, respectively, bound to DNA-cellulose (Figure 1B). It is striking that the 0.6 M NaCl eluate of the cytoplasmic fraction (Figure 1A) contained predominantly proteins specific for infected cells; virtually no ^{14}C-labeled proteins were detected in the 0.6 M eluate from uninfected cells. In addition, the 0.6 M NaCl eluate of the membrane complex had the highest ratio of ^{3}H- to ^{14}C-labeled proteins, showing that this fraction also is enriched in DNA binding proteins that are specific for infected cells.

Polyacrylamide Gel Electrophoresis of DNA Binding Proteins from the Nuclear Membrane Complex. The mixture of ^{3}H- and ^{14}C-labeled proteins eluted from DNA-cellulose by different NaCl concentrations was resolved by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. The 0.15 M eluate showed a heterogeneous pattern of polypeptides in both infected and uninfected cells (Figure 2A). The 0.4 M eluates showed a similar heterogeneous profile (Figure 2B). Two polypeptides of molecular weight 75,000 and 45,000 from infected cells could be observed in the 0.4 M NaCl eluate, although they are obscured by the background of cell-specific proteins.

A marked difference in the electrophoretic pattern of infected and uninfected cell polypeptides is evident in the 0.6 M NaCl eluate (Figure 3A). Two prominent components of average molecular weight 75,000 and 45,000, as determined in several experiments, were present in infected cell preparations (Figure 3A). These polypeptides were not detected

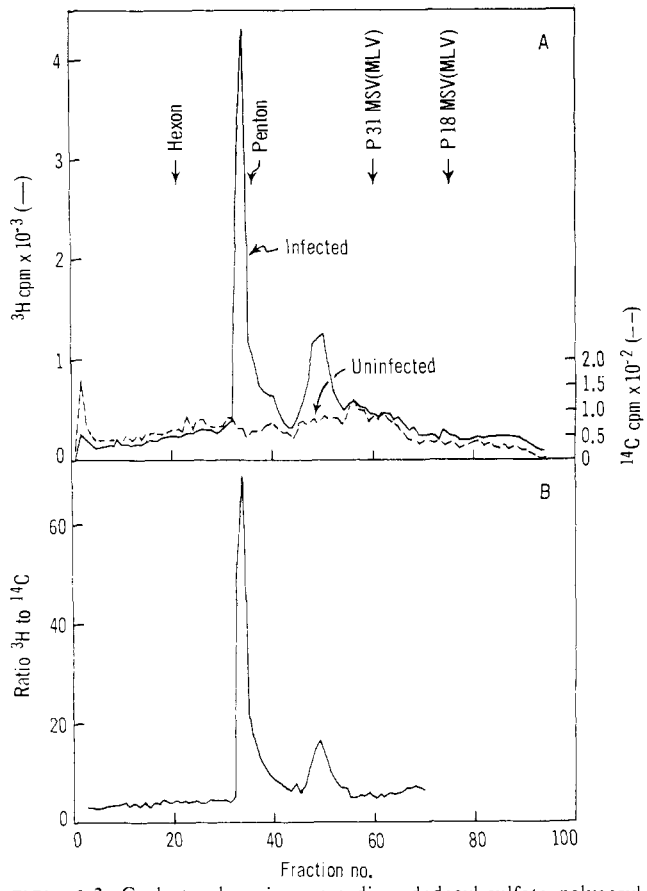

FIGURE 3: Coelectrophoresis on a sodium dodecyl sulfate-polyacrylamide gel of proteins from the membrane complex of infected ^{3}H and uninfected ^{14}C cells eluted from DNA-cellulose with 0.6 M NaCl. (A) Fractions 74-82 of Figure 1B were pooled and precipitated with Cl_3CCOOH. The precipitate (144,300 ^{3}H cpm and 18,400 ^{14}C cpm) was electrophoresed on 6% polyacrylamide gels as described under Materials and Methods. (B) The ratio of ^{3}H cpm to ^{14}C cpm in (A) is plotted.

in uninfected cell preparations which contained much less radioactivity that was distributed heterogeneously in gels (Figure 3A). The plot of the ratio of ^{3}H to ^{14}C strongly suggests that the 75,000 and 45,000 components are unique to infected cells (Figure 3B).

Figure 4 presents the electrophoretic profile of the DNA binding proteins in the 2 M NaCl eluate. A major polypeptide of molecular weight 40,000 was common to infected and uninfected cells. A second major broad peak of molecular weight 15,000–17,000 was also present in both infected and uninfected cells although higher levels were present in uninfected cells. These proteins were presumably cell-specific, since they are found in both uninfected and infected cells.

Polyacrylamide Gel Electrophoresis of DNA Binding Proteins from the Cytoplasm. The 0.15, 0.4, and 0.6 M NaCl eluates from the cytoplasm of infected and uninfected cells shown in Figure 1A were precipitated with Cl_3CCOOH and coelectrophoresed on sodium dodecyl sulfate-polyacrylamide gels. In contrast to the heterogeneous population of DNA binding proteins present in the 0.15 and 0.4 M NaCl eluates of the membrane complex from uninfected and infected cells (Figure 2), the DNA binding proteins present in the 0.15 M eluate of the cytoplasm consisted of mainly two or three peaks (Figure 5A). The major component of molecular weight 33,000 is common to infected and uninfected cells (Figure 5A) and is detected also in the

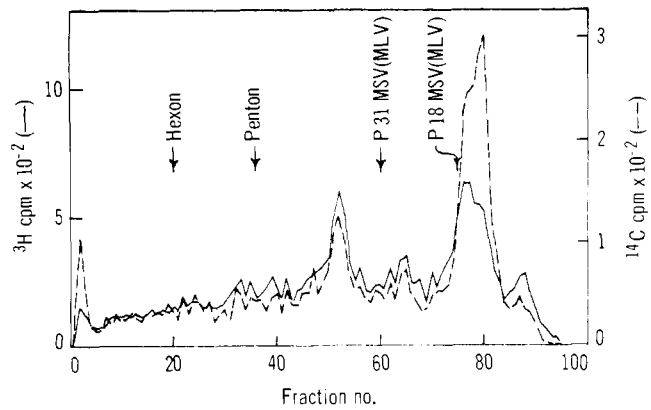

FIGURE 4: Coelectrophoresis on a sodium dodecyl sulfate-polyacrylamide gel of proteins from the membrane complex of infected ^{3}H and uninfected ^{14}C cells eluted from DNA-cellulose with 2 M NaCl. Fractions 87-93 of Figure 1B were pooled and precipitated with Cl_3CCOOH. The precipitate (56,200 ^{3}H cpm and 12,900 ^{14}C cpm) was electrophoresed on 6% polyacrylamide gels as described under Materials and Methods.

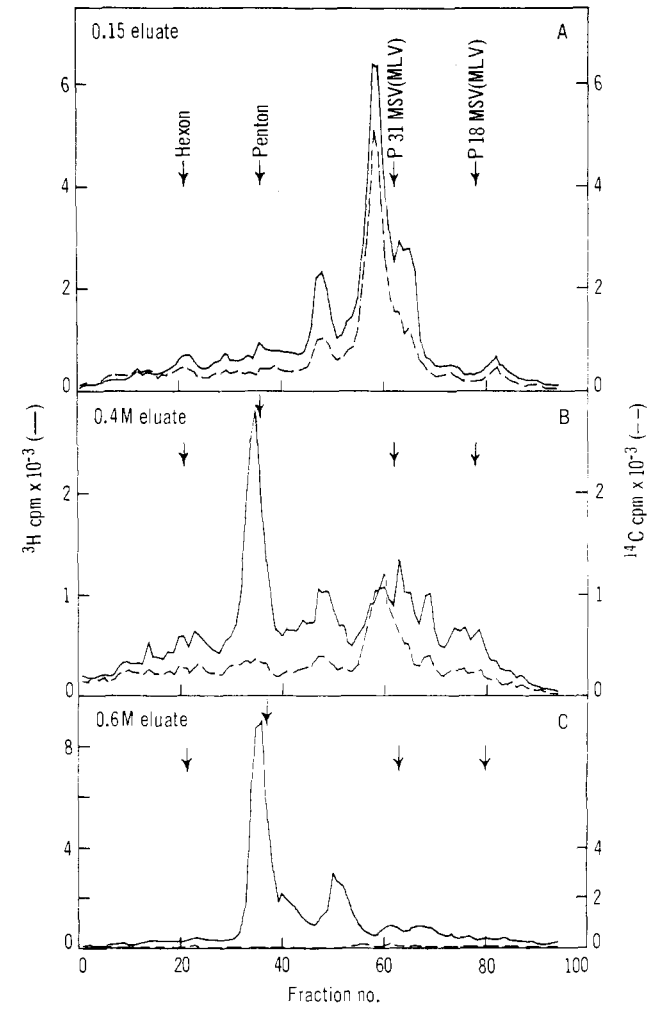

FIGURE 5: Coelectrophoresis on sodium dodecyl sulfate-polyacrylamide gels of proteins from the cytoplasm of infected ^{3}H and uninfected ^{14}C cells eluted with 0.15 M NaCl (A), 0.4 M NaCl (B), and 0.6 M NaCl (C). The NaCl eluates of Figure 1A, fractions 59-65 of the 0.15 M NaCl eluate, fractions 74-80 of the 0.4 M NaCl eluate, and fractions 90-99 of the 0.6 M NaCl eluate were pooled and precipitated with Cl_3CCOOH. The precipitates derived from 0.15 M NaCl eluate (170,600 of ^{3}H cpm and 95,000 at ^{14}C cpm, the 0.4 M eluate (149,700 ^{3}H cpm and 59,500 ^{14}C cpm), and the 0.6 M eluate (255,800 ^{3}H cpm and 8700 ^{14}C cpm) were electrophoresed on 6% polyacrylamide gels as described under Materials and Methods.

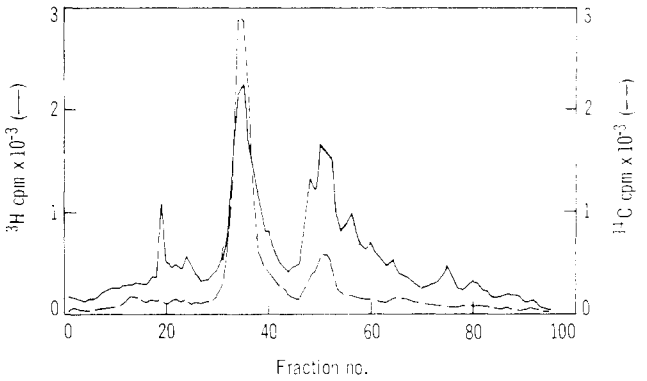

FIGURE 6: Coelectrophoresis of the DNA binding proteins present in the 0.6 M NaCl eluates of the cytoplasm and the replication complex of infected cells. The Cl_3CCOOH precipitates of the 0.6 M NaCl eluates of [^{3}H]leucine-labeled membrane complex (184,000 cpm) and [^{14}C]leucine-labeled cytoplasm (132,000 cpm) were coelectrophoresed on 6% polyacrylamide gels as described under Materials and Methods.

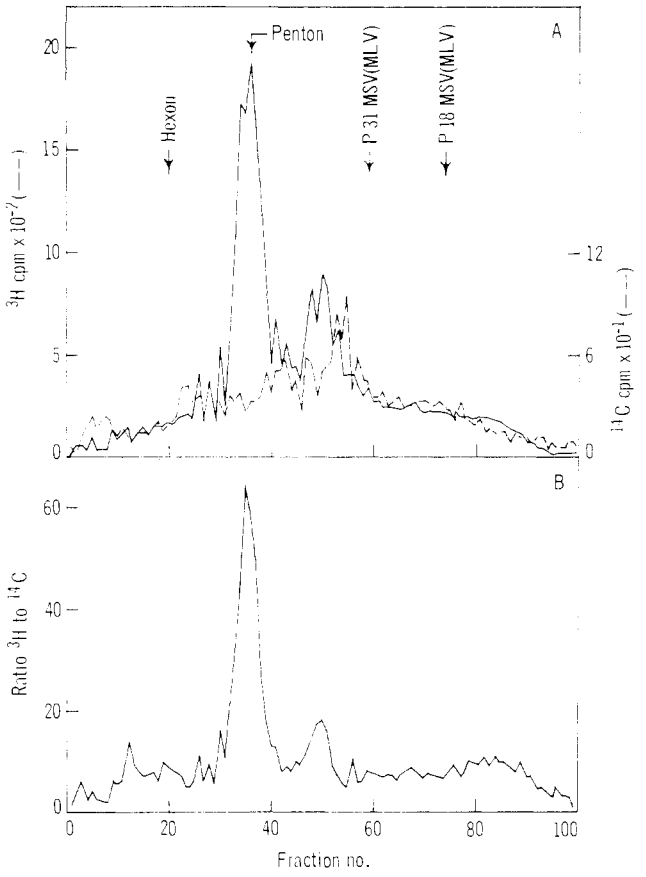

FIGURE 7: Coelectrophoresis on a sodium dodecyl sulfate–polyacrylamide gel of the DNA binding proteins present in the cytoplasm of adenovirus 2 infected ^{3}H and uninfected ^{14}C cells early after infection. (A) The DNA binding proteins in the cytoplasm of KB cells labeled with [^{3}H]leucine from 2 to 6 hr after infection with adenovirus 2 were chromatographed on DNA-cellulose. Uninfected cells were labeled with [^{14}C]leucine and processed in a similar manner. The 0.6 M NaCl eluates from infected and uninfected cell preparations were pooled and precipitated with Cl_3CCOOH. The precipitate (90,600 ^{3}H cpm and 7080 ^{14}C cpm) was analyzed by electrophoresis on 6% polyacrylamide gels as described under Materials and Methods. (B) The data are plotted as the ratio of ^{3}H to ^{14}C cpm.

0.4 M NaCl eluate (Figure 5B). The 0.4 M NaCl eluate contained large amounts of the polypeptides of molecular weight 75,000 and 45,000 that are specific for infected cells (Figure 5B). The 0.6 M NaCl eluate of the cytoplasmic fraction of infected cells consisted predominantly (about 90%) of the 75,000 and 45,000 components (Figure 5C). The 75,000 and 45,000 components were not detected in the cytoplasm of ^{14}C-labeled uninfected cells eluted from DNA-cellulose (Figure 5C). These results are consistent with the DNA-cellulose column profile in Figure 1A which detected little [^{14}C]polypeptide (uninfected cells) but considerable [^{3}H]polypeptide (infected cells) in the 0.6 M NaCl eluate.

In order to establish whether the 75,000 and 45,000 polypeptides in the cytoplasm of infected cells were identical with those in the membrane complex, the following experiment was performed. [^{3}H]Polypeptides from the membrane complex and [^{14}C]polypeptides from the cytoplasm of infected cells were isolated by elution with 0.6 M NaCl from DNA-cellulose, mixed, precipitated with Cl_3CCOOH, and coelectrophoresed (Figure 6). The ^{3}H-labeled polypeptides of molecular weight 75,000 and 45,000 comigrated with the corresponding ^{14}C-labeled polypeptides, and thus most likely represent identical molecules.

DNA Binding Proteins Present in the Cytoplasm and in Nuclear Membrane Complex Early after Infection. To determine whether the 75,000 and 45,000 molecular weight proteins were synthesized early after infection, *i.e.*, prior to the onset of viral DNA replication at 6–7 hr (Green, 1962), KB cells infected with adenovirus 2 were labeled with [^{3}H]leucine from 2 to 6 hr after infection. Uninfected cells were labeled with [^{14}C]leucine in the same manner. The DNA binding proteins were separated on DNA-cellulose columns and the 0.6 M NaCl eluate was analyzed by coelectrophoresis on polyacrylamide gels. The 0.6 M NaCl eluate of the cytoplasm contained the two polypeptides of molecular weight 75,000 and 45,000 that are specific for infected cells (Figure 7A and B). In addition, appreciable amounts of [^{3}H]polypeptides that are characteristic of uninfected cells were also present in this fraction. Labeling for longer times (6–24 hr after infection) in the presence of Ara C increases the amount of 75,000 and 45,000 polypeptides that are synthesized (Figures 5 and 6). The 75,000 and 45,000 polypeptides were also detected in the 0.6 M NaCl eluate of the membrane complex from infected cells early after infection (data not shown).

Discussion

In this paper we describe a detailed analysis of the DNA binding proteins in the cytoplasm and in a nuclear membrane complex isolated from adenovirus-infected and uninfected human KB cells. DNA binding proteins were selected by adsorption in 0.05 M NaCl to single-stranded DNA-cellulose columns and fractionated by elution with 0.15, 0.4, 0.6, and 2 M NaCl. A major fraction of the labeled proteins from the nuclear membrane complex (40–60%) and a small amount from the cytoplasm (10–20%) bound to DNA-cellulose in 0.05 M NaCl. Larger quantities of DNA binding proteins were present in the nuclear membrane complex and in the cytoplasm of infected cells than in the corresponding fractions from uninfected cells.

The electrophoretic profile of the 0.15 and 0.4 M NaCl eluates from the nuclear membrane complex revealed a heterogeneous mixture of polypeptides in infected and uninfected cells. Analysis of the 0.6 M eluate showed two major components of molecular weight 75,000 and 45,000 that were present only in infected cell preparations; these two polypeptides were not prominent in the 0.4 M eluate of the membrane complex due to the high background of cell-spe-

cific polypeptides, but were quite evident in the same eluate from the cytoplasmic fraction.

Large quantities of two DNA binding proteins of molecular weights 75,000 and 45,000 were present in the 0.4 and 0.6 M eluates of the cytoplasmic fractions of infected cells; the 0.6 M eluate consisted predominantly of these two virus-specific proteins. The identity of the DNA binding proteins in the cytoplasm and the nuclear membrane complex was established by coelectrophoresis. The DNA binding proteins of molecular weight 72,000 and 48,000 isolated from total cell extracts of monkey kidney cells abortively infected with adenovirus 5 (Van der Vliet and Levine, 1973) have similar elution characteristics and most likely correspond to the DNA binding proteins that we find in the nuclear membrane and cytoplasmic fractions of adenovirus 2 infected cells.

Two major cell-specific components of molecular weight 40,000 and 15,000–17,000 were present in the 2 M NaCl eluate of the replication complex and one major cell-specific protein of molecular weight 33,000 in the 0.15 and 0.4 M eluate of the cytoplasm of infected and uninfected cells.

The two DNA binding proteins in adenovirus 2 infected cells probably reflect the expression of the parental viral genome since (1) they are synthesized early after infection, (2) they are synthesized in the presence of Ara C which blocks the replication of adenovirus DNA, and (3) they are not detected in uninfected cells. A polypeptide of molecular weight about 70,000 has been detected in KB cells early after infection by adenovirus 2 and 5 (Russell and Skehel, 1972; Anderson *et al.*, 1973) and in HeLa cells infected by adenovirus 2 (Walter and Maizel, 1974). The synthesis of this protein was not observed late after infection at 11–15 hr (Anderson *et al.*, 1973; Walter and Maizel, 1974). Neither of these two polypeptides are present in the virion since the 75,000 and 45,000 molecular weight polypeptides in the 0.6 M eluate of the DNA cellulose column did not comigrate when coelectrophoresed with labeled adenovirus 2 proteins (unpublished data) thus confirming the results of Van der Vliet and Levine (1973).

Our studies do not establish whether the 75,000 and 45,000 molecular weight polypeptides represent viral-coded proteins or derepressed cellular proteins. One approach to establish the viral origin of these DNA binding proteins would be to synthesize them *in vitro* using early viral mRNA molecules. Recently in our laboratory Büttner *et al.* (1974) isolated early viral mRNA molecules of molecular weight 0.74×10^6 and 0.77×10^6 that are specific for the viral L- and H-DNA strand, respectively, from the polyribosomes of adenovirus 2 infected KB cells. These mRNA species are of the appropriate size to code for the DNA binding protein of molecular weight 75,000. Another approach is the isolation of mutants that are temperature sensitive in the synthesis of these proteins. Preliminary evidence for this possibility was briefly reported by Van der Vliet and Levine (1973).

The role of the 75,000 and 45,000 molecular weight proteins in viral DNA replication is not known. Their association with the nuclear membrane complex that synthesized viral DNA *in vitro* (Yamashita *et al.*, 1975) is suggestive of a function in DNA replication, although nonspecific binding cannot be ruled out. The fact that the gene 32-protein of bacteriophage T4 binds to single-stranded DNA and is required for the replication of viral DNA suggests a similar function for the DNA binding proteins in adenovirus-infected cells. Reconstruction studies using the nuclear membrane complex isolated from adenovirus infected cells and purified DNA binding proteins should define the possible role of these proteins in DNA replication.

Acknowledgments

We thank Susan Loewenstein for excellent assistance in these studies.

References

Alberts, B. M. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29,* 1154.

Alberts, B. M., and Frey, L. (1970) *Nature (London) 227,* 1313.

Anderson, C. W., Baum, P. R., and Gestland, R. F. (1973), *J. Virol. 12,* 241.

Büttner, W., Veres-Molnar, Z., and Green, M. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 2951.

Delius, H., Mantell, N. J., and Alberts, B. M. (1972), *J. Mol. Biol. 67,* 341.

Dulbecco, R., and Vogt, P. D. (1954), *J. Exp. Med. 99,* 167.

Green, M. (1962), *Cold Spring Harbor Symp. Quant. Biol. 27,* 219.

Green, M. (1970), *Annu. Rev. Biochem. 39,* 701.

Green, M., Parsons, J. T., Pina, M., Fujinaga, K., Caffier, H., and Landgraf-Leurs, I. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 803.

Kashnig, D. M., and Kasper, C. B. (1969), *J. Biol. Chem. 244,* 3786.

Litman, R. M. (1968), *J. Biol. Chem. 243,* 6222.

Maizel, Jr., J. V. (1969), *in* Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N.Y., Academic Press, p 334.

Maizel, Jr., J. V., White, D. O., and Scharff, M. D. (1968), *Virology 36,* 126.

Piña, M., and Green, M. (1969), *Virology 38,* 573.

Russell, W. C., and Skehel, J. J. (1972), *J. Gen. Virol. 15,* 45.

Shanmugam, G., Vecchio, G., Attardi, D., and Green, M. (1972), *J. Virol. 10,* 447.

Van der Vliet, P. C., and Levine, A. J. (1973), *Nature (London), New Biol. 246,* 170.

Walter, G., and Maizel, Jr., J. V. (1974), *Virology 57,* 402.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Yamashita, T., Arens, M. Q., and Green, M. (1975), *J. Biol. Chem.* (in press).

Yamashita, T., and Green, M. (1974), *J. Virol. 14,* 412.