COMPARISON OF DNA-BINDING PROTEINS FROM MOUSE CELLS IN CULTURE

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SUMMARY: DNA-binding proteins were isolated from separate clones of AKR mouse embryo cells, grown under standard conditions, by passage through DNA-cellulose columns and stepwise elution with 0.15 M, 0.6 M, and 2.0 M NaCl buffer. The eluants were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis. Incorporation of isotopic amino acid into at least one protein of the 0.15 M salt wash with a molecular weight of 80,000-90,000 was significantly lower when one clone was compared to another. Total 0.15 M NaCl eluted protein was found to inhibit the transcription of AKR/J DNA into RNA in reactions directed by *E. coli* RNA polymerase.

Recent studies have demonstrated the use of DNA-cellulose columns for the isolation of possible regulatory proteins capable of binding to DNA specifically (1, 2). Studies of DNA-binding proteins from mammalian cells have been carried out using cells grown under restrictive conditions including the omission of requisite serum growth components from the cell culture medium and the use of thymidine to cause arrest of the growth cycle (3). These studies have led us to investigate whether alterations of DNA-binding proteins occur in cell populations grown in the absence of such conditions and if these proteins can be shown to have biological function. In this communication, the results indicate that alterations of DNA-binding proteins can be detected in standard cell populations and that these proteins may block the transcription of DNA into RNA.

MATERIALS AND METHODS

Mixtures of 11 radioactive amino acids were obtained from Schwarz-Mann. The specific activity of the [^{3}H] mixture ranged from 0.6-22 C_i/mmole and the [^{14}C] from 0.16-0.31 C_i/mmole. Cultures used

were clonal lines derived from cells of the same AKR mouse embryo (4). AKR $2B^-$ is a virus-negative subclone of line 32C; AKR II $2D^+$ is a virus-producing cell line established from clone 26A which had undergone spontaneous activation and spread of type C virus. Cell lines were monitored for PPLO and infectious virus (4). Cells were grown in modified McCoy's 5A medium containing 10% fetal calf serum and antibiotics. Cell proteins were labeled using fresh media, minus all amino acids, which had been supplemented with dialyzed serum, either 3 μC_i/ml of [^{14}C] or 30 μC_i/ml of [^{3}H] amino acid mixture, and unlabeled amino acids consisting of L-histidine, L-tyrosine, glycine, L-proline, L-cystine, L-phenylalanine, L-glutamine, and L-asparagine at a concentration each of either 29 µM or 180 µM for the [^{14}C] or [^{3}H] medium labeling mixture respectively. Cells were incubated for 24 hrs and then washed, harvested, and suspended in homogenization medium containing 0.04 M Tris-HCl, pH 8.1, 0.2 M EDTA, 0.01 M $MgCl_2$, 5.0 M NaCl, and 0.001 M DTT. Homogenization was carried out in a Sorvall Omni-Mixer for 30 seconds at maximal speed followed by sonication at maximum frequency for 30 seconds. These and all subsequent procedures were carried out at 0 - 4° unless otherwise noted. The homogenate was fractionated with a dextran-polyethylene glycol two-phase system (5) in order to separate the proteins from nucleic acids. The upper phase containing polyethylene glycol was dialyzed against 1,000 volumes of buffer containing 0.02 M Tris-HCl, pH 8.1, 0.001 M EDTA, 0.005 M NaCl, and 0.001 M DTT. The [^{3}H] and [^{14}C] labeled cell extracts were mixed together and passed through a DNA-cellulose column prepared according to Alberts et al. (1), with modification for mammalian cell studies (2). Mouse DNA was prepared from 14-17 day-old AKR/J mouse embryos (6). DNA-binding proteins were eluted (2) and lyophilized after exhaustive dialysis. Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis run at room temperature (2). Gels were cut manually into 2mm slices and incubated for 1 hr at 80° in 0.2 ml

of 0.5 M NaOH. After standing overnight at 25°, 10 ml of scintillation fluid containing 58% toluene, 32% Triton X-100, 4% Liquifluor, 2% HCl, and 4% water (v/v) was added. Samples were counted in a Beckman liquid scintillation spectrometer at an efficiency of 26% for [^{3}H] and 63% for [^{14}C].

RESULTS

It was previously reported that alterations in specific proteins binding to DNA-cellulose columns occurred with mouse cells in transition between the growing and resting state (2). In order to eliminate the possibility that alterations in DNA-binding proteins were due to differences in rates of growth between clones, cell growth curves were determined for both the AKR 2B^{-} and AKR II 2D^{+} cell lines. The results are presented in Fig. 1. Cells at approximately the same density in late log phase were labeled with isotopic amino acids for 24 hrs. The proteins binding to the DNA-cellulose column were selectively eluted at 0.15, 0.6 and 2.0 M NaCl. The polyacrylamide gel profiles for each fraction are presented in Fig. 2. In contrast to the 0.6 M and 2.0 M NaCl eluted fractions, only the 0.15 M NaCl fractions contained a peak by gel chromatography which was significantly greater in the virus-negative cell line than in the clone-producing virus. Similar results were obtained in two additional but separate experiments with these cell lines. This protein was estimated to have a molecular weight of 80,000-90,000 daltons in sodium dodecyl sulfate polyacrylamide gels (Fig. 3). When the AKR II 2D^{+} cell line was incubated with the [^{3}H] amino acid mixture instead of the [^{14}C] one, similar results were obtained. When the DNA-cellulose column was washed for 24 hrs at 24°, instead of 0-4°, prior to eluting the proteins off with NaCl buffer, peak P_1 was still detected in the 0.15 M eluant.

Nonspecific binding of this protein to the cellulose matrix of the DNA-cellulose column appears unlikely. In separate studies using conditions similar to those of this report, less that 0.2% of the total

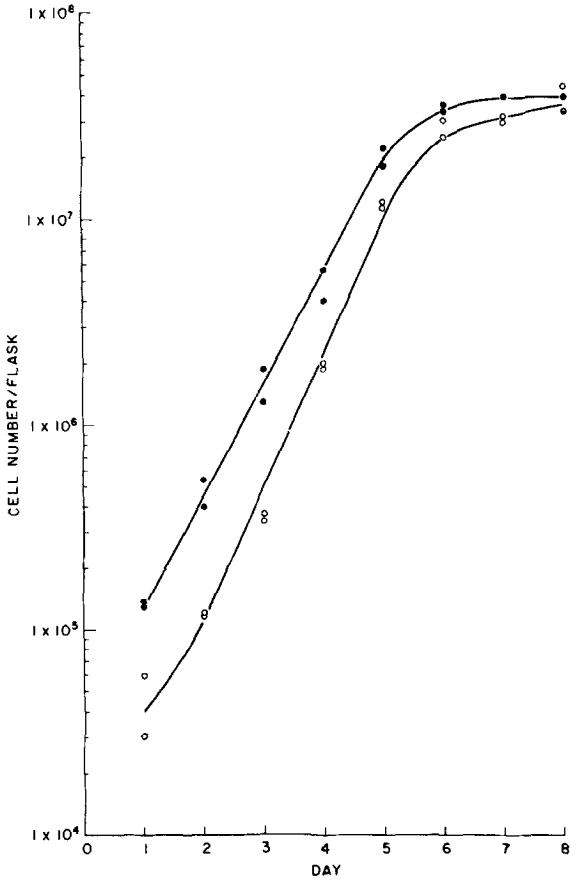

Fig. 1. Growth of AKR 2B⁻ and AKR II 2D⁺ cell lines. Falcon flasks (75 cm^2) were seeded with either 2.5 x 10^4 cells (AKR 2B⁻) or 7.5 x 10^4 cells (AKR II 2D⁺) in 13 ml medium. Medium was changed on days 1, 3 and 5. At 24 hr intervals, two flasks of each cell line were treated with a trypsin-versene mixture to release the cells which were then counted. ∘—∘—∘, AKR 2B⁻; •—•—•, AKR II 2D⁺.

radioactivity bound to columns in the absence of DNA (2, 3).

The possibility that peak P_1 is a growth regulatory protein is also unlikely. Relatively minor alterations in proteins corresponding to the position for peak P_1 in SDS-polyacrylamide gels have been reported for a mouse fibroblast cell line within 12 hrs after release from serum deprivation. No changes were noted after 12-18 hrs (2). Another minor alteration was observed for Chinese hamster ovary cell lines when the proteins from synchronized cells made during the S and G_1 phase of cell

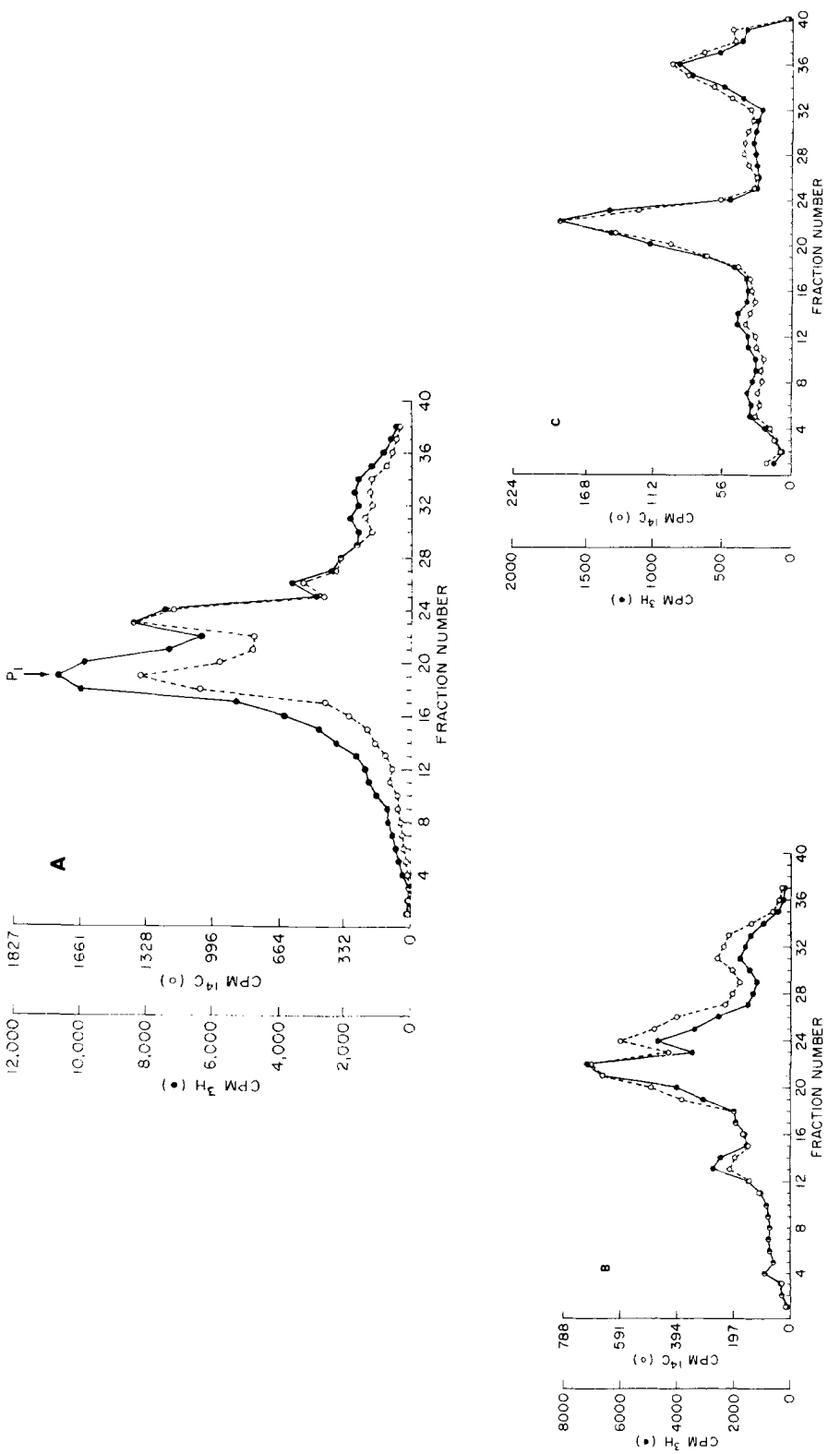

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of labeled DNA-binding proteins. (A) Coelectrophoresis of proteins of 0.15 M NaCl eluates obtained from ^{3}H-labeled AKR 2B^{-} and ^{14}C-labeled AKR II 2D^{+} cells. (B) Coelectrophoresis of proteins of 0.6 M NaCl eluates. (C) Coelectrophoresis of proteins of 2.0 M NaCl eluates.

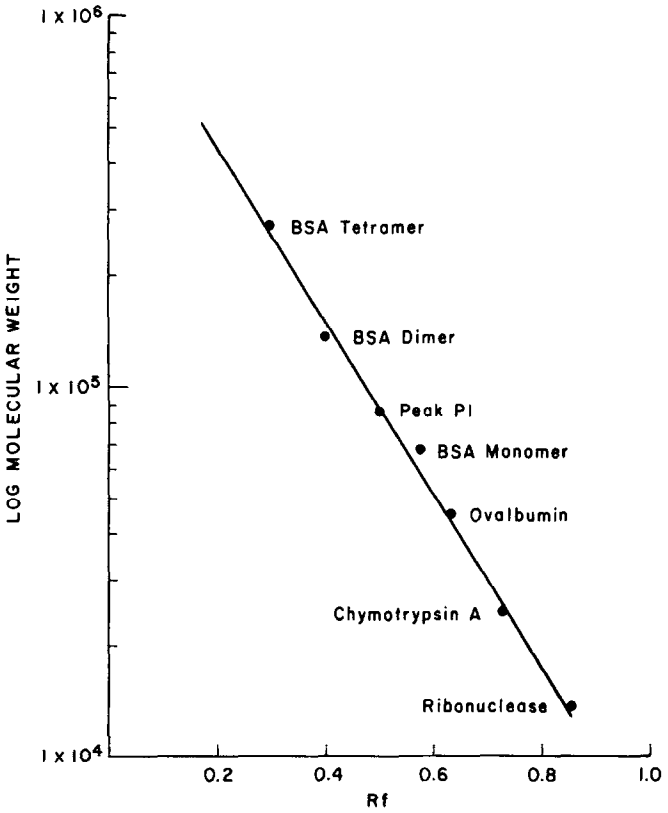

Fig. 3. Calibration of the molecular weight of peak P_1. Samples containing 50 µg each of purified protein were run in sodium dodecyl sulfate polyacrylamide gels as described in Methods. The gels were stained with 0.2% coomassie blue in a 50% methanol solution and destained in 7% acetic acid.

growth were compared (3). In contrast, cells in this study were labeled and harvested from mixed phase cell populations at equivalent stages of growth, in the absence of serum deprivation.

Peak P_1 does not appear to correspond to another class of proteins found in mammalian cells which are also capable of interacting with DNA. Such proteins, termed histones, generally have molecular weights below 35,000, contain little or no tryptophan, and are basic. Earlier studies have already demonstrated, using both bacterial and mammalian systems, that nonhistone, nonbasic proteins are eluted from DNA-cellulose under the conditions of these studies (1, 2).

If the 0.15 M salt fraction proteins are involved in some manner with the endogenous expression of type C viral genetic information, there must be a basis for biological activity. Tests of this fraction from two separate experiments for deoxyribonuclease or ribonuclease activity, using 10,225 cpm of either [^{14}C] labeled mouse DNA (7) or [^{3}H] labeled mouse ribosomal RNA (8) and incubation for up to 40 min. at 37° were negative. The 0.15 M salt eluant proteins also did not demonstrate either detectable RNA polymerase (9) or DNA polymerase (10) activity. However, regulatory proteins from bacterial systems known to block transcription *in vitro* exhibit a considerable reduction in binding to DNA in the presence of 0.15-0.2 M salt (11, 12). This suggested that at least one of the proteins in the 0.15 M NaCl fraction from mouse cells might be also capable of altering transcription in an *in vitro* system. It was found that *E. coli* RNA polymerase activity could be inhibited more than 50% by approximately 4 µg or less of 0.15 M salt wash protein when 15 µg of AKR/J DNA was used as a template (9). Controls showed that neither polyethylene glycol (3.5-35 mg/ml) or 0.15 M NaCl elution buffer (1:10 dilution) produced detectable inhibition of polymerase activity. Crystallized bovine serum albumin at a concentration of 5 mg/ml also had no effect. At the present time, it cannot be determined which proteins in this fraction are responsible for the observed activity.

The 0.6 M salt wash proteins were not assayed due to the presence of interfering RNA and DNA polymerase activities (1). Limiting amounts of the 2.0 M fraction made their assay difficult. Conceivably, both of these fractions may also contain proteins capable of inhibiting transcription *in vitro*.

Studies are presently in progress to utilize newly developed cell lines in order to determine whether the alterations of peak P_1 observed in this report are due to normal differences between clones unrelated to endogenous viral expression or to the presence of replicating AKR virus

in one clone and not in the other.

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