Design and Preparation of Affinity Columns for the Purification of Eukaryotic Messenger Ribonucleic Acid Cap Binding Protein†

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ABSTRACT: 2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]-7-methylguanosine 5′-diphosphate (**5**) and 7-(5-carboxypentyl)guanosine 5′-diphosphate (**13**) have been synthesized and immobilized on AH-Sepharose 4B to the extent of 17.4 and 36.6 μmol of ligand/g of gel, respectively. The affinity resins thus derived were employed in columns for the purification of 24K cap binding protein (CBP) from rabbit reticulocytes. Each resin was found to retain the protein of interest; elution of 24K CBP could then be effected by washing with 70 μM m^{7}GDP. The 24K CBPs released from both columns were found to be active, both as judged by a cross-linking assay that utilized IO_4-oxidized methyl-^{3}H-labeled reovirus mRNA as a substrate for the protein and also by the ability of the isolated 24K CBP to stimulate the translation of capped Sindbis virus mRNA in HeLa cell extracts.

Most eukaryotic mRNAs have a 5′-terminal "cap" structure, m^{7}GpppN, that distinguishes them structurally from prokaryotic mRNAs (Shatkin, 1976). The mRNA caps serve to stabilize mRNAs (Furuichi et al., 1977; Shimotohno et al., 1977; Gedamu & Dixon, 1978) and facilitate initiation of translation (Shatkin, 1976). Recognition of the cap structure by eukaryotic initiation factors has been studied; rabbit reticulocytes as well as mouse L and ascites cells have been shown (Sonenberg et al., 1978) to contain a protein of apparent M_r 24000 that binds specifically to capped mRNAs from several species. This cap binding protein (24K CBP)[1] stimulated the in vitro translation in HeLa cell extracts of capped mRNAs (e.g., Sindbis and reovirus mRNAs), but not of the naturally uncapped mRNAs derived from encephalomyocarditis virus and satellite tobacco necrosis virus (Sonenberg et al., 1979). The 24K CBP was also found to mediate differential translation of capped mRNAs in the presence of competing uncapped species (Sonenberg et al., 1980). Furthermore, inactivation of cap binding protein function may explain the shut off of host mRNA translation in poliovirus-infected cells (Trachsel et al., 1980).

Chemical studies of 7-alkylguanosine derivatives structurally related to mRNA caps have suggested the existence of a preferred conformation that may provide the basis for mRNA cap recognition by cap binding proteins (Hickey et al., 1977; Adams et al., 1978). The ability of a number of cap analogues to inhibit the formation of an initiation complex between reovirus mRNA and wheat germ ribosomes has afforded additional information that may be pertinent to the recognition of mRNA caps by specific proteins. The accumulated data has provided the basis for design of affinity ligands of potential utility for the purification of such proteins. In the present report, we describe the preparation of affinity ligands, their immobilization on AH-Sepharose 4B, and experiments that define conditions under which the affinity resins can be employed for the purification of cap binding proteins.

Experimental Procedures

Materials. Guanosine, 1,1′-carbonyldiimidazole, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride were obtained from Sigma Chemical Co., as were Sephadex LH-20 and AH-Sepharose 4B. DEAE-cellulose (DE-23) was purchased from Whatman; (Macherey-Nagel and Co.) poly(ethylenimine) TLC sheets were from Brinkmann Instruments.

Physical Measurements. Melting points were determined on a Thomas Hoover apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 457A spectrophotometer, and ultraviolet and visible spectra were recorded on a Cary 15 spectrophotometer. Mass spectra were obtained by using a Varian MAT-44 spectrophotometer. ^{1}H Nuclear magnetic resonance (NMR) spectra were measured on Varian T-60 and Hitachi Perkin-Elmer R-22 (90 MHz) instruments and are given in parts per million (δ) downfield from tetramethylsilane as an internal or external standard, as specified. Fluorescence spectra were determined on a Turner Model 430 Spectrofluorometer.

*Synthesis of Ligands. 2′,3′-O-[1-(3-Ethoxy-3-oxopropyl)ethylidene]guanosine 5′-Monophosphate (**2**).* To a suspension of 0.316 g (0.771 mmol) of 2′,3′-O-[1-(3-ethoxy-3-oxopropyl)ethylidene]guanosine (**1**) (Seela & Waldek, 1975) in 10 mL of acetonitrile at 0 °C was added 0.62 mL (0.971 g, 3.86 mmol) of pyrophosphoryl chloride (Crofts et al., 1960). The resulting solution was stirred at 0 °C for 2 h and then neutralized by the addition of 15 mL of saturated aqueous $NaHCO_3$. The solution was concentrated under diminished pressure to remove CH_3CN and was then diluted to 50 mL with water and treated with 16 mL of 0.25 M aqueous barium acetate. The suspension was centrifuged, and the solid residue was washed twice with water. The combined supernatant was diluted to 400 mL and purified (in two portions) by successive chromatographies on DEAE-cellulose columns (2.4 × 33 cm; HCO_3^- form); elution was at 4 °C with a linear gradient of ammonium bicarbonate (0–0.20 M; 2 L total volume; 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 7300 A_{253} units

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[1] Abbreviations used: CBP, cap binding protein; AH-Sepharose 4B, aminohexyl-Sepharose 4B; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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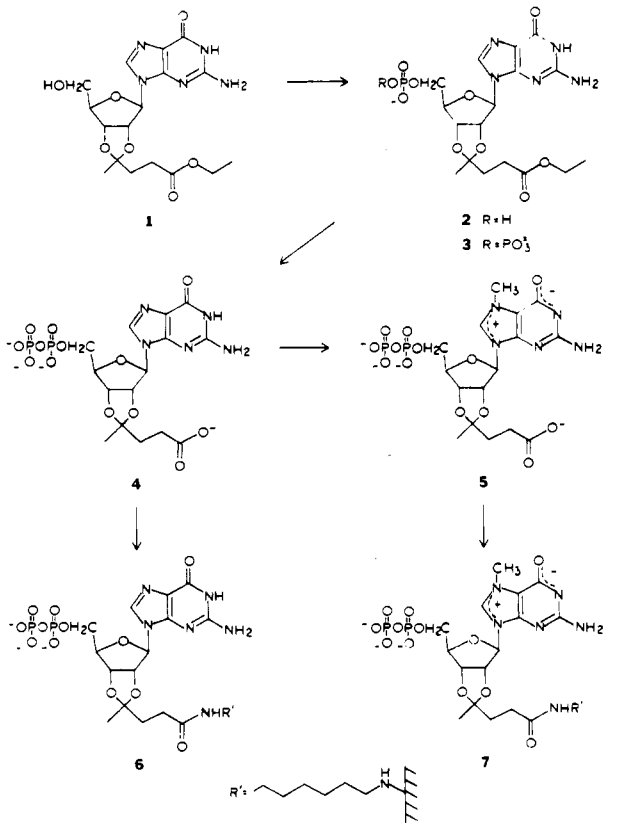


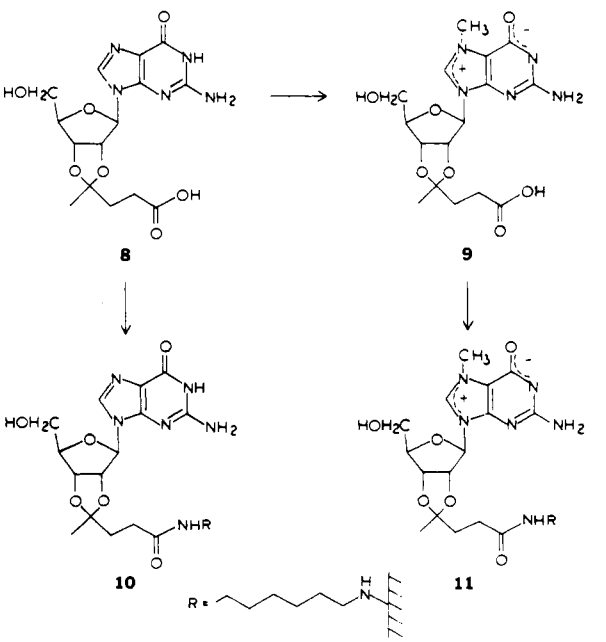
(71%) of the monophosphate: $\lambda_{max}^{H_2O}$ (pH 1) 255, 277 (sh); λ_{max} (pH 7.5) 253, 272 (sh); λ_{max} (pH 13) 262 nm; NMR [D_2O, external $(CH_3)_4Si$] δ 1.27 (t, 3 H, J = 7 Hz), 1.33 (s, 23 H), 1.47 (s, 0.7 H) 2.33 (t, 2 H), 2.58 (t, 2 H), 4.13 (m, 4 H), 4.56 (s, br, 1 H), 5.20 (s, br, 1 H), 5.38 (s, br, 1 H), 6.11 (s, br, 1 H); TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.53.

2',3'-O-[1-(3-Ethoxy-3-oxopropyl)ethylidene]guanosine 5'-Diphosphate (**3**). To 2670 A_{253} units (0.206 mmol) of 2',3'-*O*-[1-(3-ethoxy-3-oxopropyl)ethylidene]guanosine 5'-monophosphate (**2**) as the anhydrous mono(tri-*n*-hexylammonium) salt was added 0.186 g (1.15 mmol) of 1,1'-carbonyldiimidazole and 2 mL of anhydrous *N*,*N*-dimethylformamide. The solution was stirred under N_2 for 24 h and then treated with 38 μL of dry methanol. After 30 min, a solution containing 1.13 mmol of anhydrous mono(tri-*n*-hexylammonium) phosphate in 2 mL of dry dimethylformamide was added dropwise with vigorous stirring, and the combined solution was stirred at 25 °C (N_2) for an additional 24 h. The solution was concentrated under diminished pressure, and the residue was suspended in 150 mL of water and applied to a DEAE-cellulose column (2.4 × 33 cm; HCO_3^- form) that was washed with 10 mL of methanol and 75 mL of water. The column was then washed at 4 °C with a linear gradient of ammonium bicarbonate (0–0.4 M, 2 L total volume, 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 2160 A_{253} units (78%) of the desired nucleoside 5'-diphosphate (**3**) as a clear glass: $\lambda_{max}^{H_2O}$ (pH 1) 255, 277 (sh); λ_{max} (pH 7.5) 253, 272 (sh); λ_{max} (pH 13) 262 nm; NMR [D_2O, external $(CH_3)_4Si$] δ 1.23 (t, 3 H, J = 7), 1.39 (s, 2.1 H), 1.53 (s, 0.9 H), 2.33 (m, 2 H), 2.55 (m, 2 H), 3.86–4.67 (m, 5 H), 5.1–5.27 (m, 2 H), 5.98 (s, br, 1 H), 7.93 (s, br, 1 H); TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.35.

2',3'-O-[1-(2-Carboxyethyl)ethylidene]guanosine 5'-Diphosphate (**4**). A solution of 3555 A_{253} units (0.265 mmol) of 2',3'-*O*-[1-(3-ethoxy-3-oxopropyl)ethylidene]guanosine 5'-diphosphate was dissolved in 10 mL of 0.5 M aqueous NaOH. The solution was stirred at 0 °C for 30 min and then diluted with 50 mL of water and neutralized (pH 6.5) with 1 M HCl. The solution was diluted to 250 mL and purified by chromatography at 4 °C on a column of DEAE-cellulose (2.4 × 33 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.6 M; 2 L total volume, 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 3350 A_{253} units (95%) of guanosine 5'-diphosphate derivative **4** as a clear glass: $\lambda_{max}^{H_2O}$ (pH 1) 254, 277 (sh); λ_{max} (pH 7.5) 253, 273 (sh); λ_{max} (pH 13) 262 nm; NMR [D_2O, external $(CH_3)_4Si$] δ 1.42 (s, 2.2 H), 1.60 (s, 0.8 H), 2.00–2.65 (m, 4 H), 4.0–4.4 (m, 2 H), 4.5–4.8 (m, 1 H), 5.0–5.5 (m, 2 H), 6.03 (s, br, 1 H), 7.97 (s, br, 1 H); TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.25.

2',3'-O-[1-(2-Carboxyethyl)ethylidene]-7-methylguanosine 5'-Diphosphate (**5**). To a suspension of 1000 A_{253} units (74.6 μmol) of nucleoside 5'-diphosphate **4** in 3 mL of dry dimethyl sulfoxide was added 100 μL of dry dioxane containing 7 M hydrogen chloride and 1.0 mL (2.28 g, 16.1 mmol) of methyl iodide. The solution was stirred at room temperature for 4 h, then neutralized with 2 mL of saturated aqueous $NaHCO_3$, and diluted to 25 mL with water. The solution was extracted with three 15-mL portions of ether, and the aqueous phase was diluted to 200 mL and applied to a column of DEAE-cellulose (2.4 × 33 cm; HCO_3^- form). The column was washed with 150 mL of water and then with a linear gradient of ammonium bicarbonate (0–0.4 M, 2 L total volume, 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 510 A_{258} units (pH 7.5; 76%) of a fluorescent glass: $\lambda_{max}^{H_2O}$ (pH 1) 257, 277 (sh); λ_{max} (pH 7.5) 257, 279; λ_{max} (pH 13) 268 nm; fluorescence spectrum (pH 1) λ_{ex} 295, λ_{em} 375; (pH 7.5) λ_{ex} 288, λ_{em} 391; (pH 13) λ_{ex} 303, λ_{em} 375 nm; NMR [D_2O, external $(CH_3)_4Si$] δ 1.46 (s, 2.2 H) 1.61 (S, 0.8 H), 2.2 (m, 2 H), 2.47 (m, 2 H), 4.0–4.3 (m, 5 H), 4.89 (m, 1 H), 5.11–5.49 (m, 2 H), 6.20 (s, br, 1 H); TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.75, [0.5 M LiCl, poly(ethylenimine)] R_f 0.1.

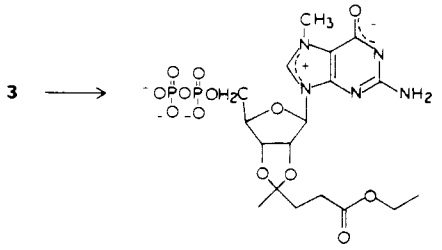
2',3'-O-[1-(2-Carboxyethyl)ethylidene]-7-methylguanosine (**9**). To a solution of 0.024 g (64.0 μmol) of 2',3'-*O*-[1-(2-



carboxyethyl)ethylidene]guanosine (**8**) (Seela & Waldek, 1975) in 1 mL of dry dimethyl sulfoxide was added 400 μL (0.912 g, 6.42 mmol) of methyl iodide. The solution was

stirred at room temperature for 4 h and then diluted with 1 mL of ethanol and 20 mL of petroleum ether. The solution was decanted from the oily residue, which was dissolved in 25 mL of water and purified by chromatography on DEAE-cellulose (2 × 20 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.15 M, 1 L total volume, 8-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 242 A_{258} units (pH 7.5, 42%) of 2′,3′-*O*-[1-(2-carboxyethyl)-ethylidene]-7-methylguanosine as the ammonium salt: $\lambda_{max}^{H_2O}$ (pH 1) 257, 277 (sh); λ_{max} (pH 7.5) 257, 280; λ_{max} (pH 13) 266 nm; fluorescence spectrum (pH 1) λ_{ex} 298, λ_{em} 375; (pH 7.5) λ_{ex} 298, λ_{em} 393; λ_{ex} 301, λ_{em} 375 nm; TLC [0.5 M LiCl, poly(ethylenimine)] R_f 0.85.

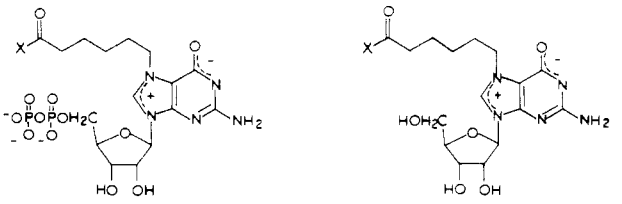
2′,3′-O-[1-(3-Ethoxy-3-oxopropyl)ethylidene]-7-methylguanosine 5′-Diphosphate (**12**). To a solution of 200 A_{253}



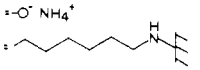
12

units (14.9 μmol) of 2′,3′-*O*-[1-(3-ethoxy-3-oxopropyl)-ethylidene]guanosine 5′-diphosphate in 1.0 mL of anhydrous dimethyl sulfoxide was added 10 μL of dry dioxane containing 7 M hydrogen chloride and 400 μL (0.912 g, 6.42 mmol) of methyl iodide. The solution was stirred at room temperature for 4 h and was then partitioned between ether and water. The aqueous layer was extracted with two 5-mL portions of ether and then purified by chromatography on DEAE-cellulose (2 × 20 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.4 M, 1 L total volume, 7-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 95 A_{258} units (71%) of nucleoside 5′-diphosphate **12** as a colorless glass: $\lambda_{max}^{H_2O}$ (pH 1) 257, 277 (sh); λ_{max} (pH 7.5) 258, 279; λ_{max} (pH 13) 268 nm; fluorescence spectrum (pH 1) λ_{ex} 299, λ_{em} 378; (pH 7.5) λ_{ex} 294, λ_{em} 391; (pH 13) λ_{ex} 298, λ_{em} 370 nm; TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.87, [0.5 M LiCl, poly(ethylenimine)] R_f 0.32.

7-(5-Carboxypentyl)guanosine 5′-Diphosphate (**13**). A



13 X = $-O^- NH_4^+$ **14**

15 X =  **16**

17 X = OCH_2CCl_3 **18**

solution containing 2770 A_{253} units (0.206 mmol) of triammonium guanosine 5′-diphosphate and 3.90 g (16.1 mmol) of 6-iodohexanoic acid in 10 mL of dimethyl sulfoxide and 1 drop of 1 M hydrochloric acid was stirred at room temperature for 48 h. The solution was partitioned between ether and water, and the aqueous layer was extracted with three 25-mL portions of ether. The product was purified by chromatography at 4 °C on DEAE-cellulose (2.4 × 33 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.4 M, pH 8.0, 2 L total volume, 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted; analysis of this material indicated that it was a mixture of GDP and **13**. This material was mixed with an additional 1200 A_{253} units of guanosine 5′-diphosphate and 4.52 g (18.2 mmol) of 6-iodohexanoic acid and stirred (25 °C, 48 h) in 5 mL of dimethyl sulfoxide that had been acidified by the addition of 1 drop of 1 M hydrochloric acid. The reaction mixture was worked up as before and purified by chromatography at 4 °C on DEAE-cellulose (2.4 × 33 cm; HCO_3^- form, pH 7.4) with a linear gradient of ammonium bicarbonate (0–0.4 M, 2 L total volume, 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 1040 A_{250} units (50%, 82% based on consumed starting material) of nucleoside 5′-diphosphate **13** as a fluorescent glass: $\lambda_{max}^{H_2O}$ (pH 1) 257, 277 (sh); λ_{max} (pH 7.5) 255, 278; λ_{max} (pH 13) 267 nm; fluorescence spectrum (pH 1) λ_{ex} 298, λ_{em} 375; (pH 7.5) λ_{ex} 297, λ_{em} 406; (pH 13) λ_{ex} 311, λ_{em} 406 nm; NMR [D_2O, external $(CH_3)_4Si$] δ 1.1–2.4 (m, 8 H), 3.9–4.6 (m, 7 H), 5.95 (d, 1 H, J = 4 Hz); TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.78, [0.5 M LiCl, poly(ethylenimine)] R_f 0.1.

7-(5-Carboxypentyl)guanosine (**14**). A solution of 0.204 g (0.720 mmol) of guanosine and 1.757 g (7.20 mmol) of 6-iodohexanoic acid in 2 mL of dimethylacetamide and 2 mL of dimethyl sulfoxide was stirred at room temperature for 24 h. The solution was concentrated and then diluted with 10 mL of ethanol and 14 mL of petroleum ether. The solvent was decanted, and the oily residue was triturated with 6 mL of acetone. After filtration, the filtrate was concentrated under diminished pressure, and the residue was purified by chromatography on DEAE-cellulose (2 × 20 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.2 M; 1 L total volume, 10-mL fractions) at a flow rate of 190 mL/h. The appropriate fractions were pooled and desalted to afford 1040 A_{260} units (17%) of 7-(5-carboxypentyl)guanosine (**14**) as a clear glass: $\lambda_{max}^{H_2O}$ (pH 1) 257, 277 (sh); λ_{max} (pH 7.5) 256, 282; λ_{max} (pH 13) 26 nm; fluorescence spectrum (pH 1) λ_{ex} 301, λ_{em} 387; (pH 7.5) λ_{ex} 311, λ_{em} 404; (pH 13) λ_{ex} 299, λ_{em} 408 nm; NMR [D_2O, external $(CH_3)_4Si$] δ 1.1–2.4 (m, 8 H), 3.9 (m, 2 H), 4.1–4.8 (m, 5 H), 6.0 (d, 1 H, J = 4 Hz); TLC [0.5 M LiCl, poly(ethylenimine)] R_f 0.87.

7-[6-(2,2,2-Trichloroethoxy)-6-oxohexyl]guanosine 5′-Diphosphate (**17**). A solution of 2500 A_{253} units (0.186 mmol) of triammonium guanosine 5′-diphosphate and 3.5 mL (14.1 mmol) of 2,2,2-trichloroethyl 6-iodohexanoate in 5 mL of dimethyl sulfoxide and 1 drop of 1 M HCl was stirred at room temperature for 48 h. The solution was diluted to 40 mL with water and extracted with three 15-mL portions of ether. The aqueous layer was diluted to 200 mL, and the product was purified by chromatography at 4 °C on DEAE-cellulose (2.4 × 35 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.4 M, 2 L total volume, 15-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled and desalted to afford 505 A_{258} units (31%, pH 7.5) of ribonucleoside 5′-diphosphate **17** as a fluorescent glass: $\lambda_{max}^{H_2O}$ (pH 1) 257, 277 (sh); λ_{max} (pH 7.5) 256, 279; λ_{max} (ph 13) 267 nm; fluorescence spectrum (pH 1) λ_{ex} 305, λ_{em} 388; (pH 7.5) λ_{ex} 294, λ_{em} 406; (pH 13) λ_{ex} 295, λ_{em} 360 nm; TLC [1.2 M LiCl, poly(ethylenimine)] R_f 0.75, [0.5 M LiCl, poly(ethylenimine)] R_f 0.4.

7-[6-(2,2,2-Trichloroethoxy)-6-oxohexyl]guanosine (**18**). To a solution of 77 mg (0.272 mmol) of guanosine in 5 mL

of dimethyl sulfoxide was added 600 μL (0.241 mmol) of 2,2,2-trichloroethyl 6-iodohexanoate. The solution was stirred at room temperature for 72 h and then partitioned between ether and water. The aqueous layer was extracted with two 15-mL portions of ether and then diluted to 100 mL, and the product was purified by chromatography on DEAE-cellulose (2 × 23 cm; HCO_3^- form); elution was with a linear gradient of ammonium bicarbonate (0–0.1 M, 1 L total volume, 8-mL fractions) at a flow rate of 180 mL/h. The appropriate fractions were pooled, desalted, and applied to a column of Sephadex LH-20 (2 × 25 cm). The column was washed with water, and the appropriate fractions were pooled and desalted to afford 770 A_{260} units (32%) of guanosine nucleoside **18** as a homogeneous, fluorescent solid: $\lambda_{max}^{H_2O}$ (pH 1) 257, 274 (sh); λ_{max} (pH 7.5) 258, 278; λ_{max} (pH 13) 267 nm; fluorescence spectrum (pH 1) λ_{ex} 303, λ_{em} 378; (pH 7.5) λ_{ex} 308, λ_{em} 405; (pH 13) λ_{ex} 298, λ_{em} 375 nm; TLC [0.5 M LiCl, poly(ethylenimine)] R_f 0.87.

6-Iodohexanoic Acid. To a solution of 14.33 g (95.6 mmol) of sodium iodide in 50 mL of dry acetone was added 6.17 g (31.6 mmol) of 6-bromohexanoic acid. A white precipitate formed quickly, and the mixture was stirred under nitrogen at 25 °C. After 24 h, the solution was filtered, and the filtrate was concentrated. The residue was partitioned between ether and water, and the ether layer was extracted with 5% aqueous $Na_2S_2O_3$ and water. The dried (Na_2SO_4) ether extract was concentrated under diminished pressure to afford a white solid. Recrystallization from pentane afforded 6.89 g (90%) of 6-iodohexanoic acid as colorless needles: mp 45 °C; IR (NaCl) 3400 (br, -OH), 2945, 2870, 1705 (-COOH dimer), 1650, 1465, 1430, 1415, 1205, 1170, 1120 cm^{-1}; NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.67 (m, 6 H), 2.35 (t, 2 H, J = 6 Hz), 3.18 (t, 2 H, J = 7 Hz), 11.50 (s, 1 H, ex); mass spectrum, *m/e* 242 (M^+), 255, 169, 155, 141, 127 (I^+), 115 (M^+–I), 97, 73, 69, 55, 41.

2,2,2-Trichloroethyl 6-Iodohexanoate. To a solution of 9.37 g (38.7 mmol) of 6-iodohexanoic acid and 6.36 g (42.6 mmol) of 2,2,2-trichloroethanol in dry methylene chloride was added 9.47 g (45.9 mmol) of *N,N'*-dicyclohexylcarbodiimide. The mixture was stirred overnight at 25 °C and then filtered, and the filtrate was concentrated. Chromatography on a column of silica gel (elution with 10% ether in pentane) afforded 12.93 g (90%) of a pale yellow liquid: IR (NaCl plates) 2940, 2870, 1750, 1455, 1430, 1380, 1220, 1150, 1125, 1055, 800, 720 cm^{-1}; NMR [$CDCl_3$, $(CH_3)_4Si$] δ 1.67 (m, 6 H), 2.50 (t, 2 H, J = 7 Hz), 3.17 (t, 2 H, J = 6 Hz), 4.80 (s, 2 H); mass spectrum, *m/e* 373, [341, 339, 337] (M^+–HCl), [251, 249, 247, 245] (0.04:0.30:0.90:1, M^+–HI), 225 [M^+–(OCH_2CCl_3)], 206, 205, 191, 163, 143, 124, 97, 83, 69, 55, 41.

Preparation of Affinity Resins. Immobilization of the ligands on AH-Sepharose 4B was carried out by modification of the method of Seela & Waldek (1975). AH-Sepharose 4B (0.250 g) was swollen in 10 mL of 0.5 M NaCl for 2 h and then washed on a fritted glass filter with 50 mL of 0.5 M NaCl and then with 50 mL of H_2O. A 1.0-mL sample of the wet resin was treated with the appropriate ligand (11.1 μmol dissolved in 2 mL of water),[2] and the pH was adjusted to 5.5–6.0 with $NaHCO_3$. The suspension was shaken gently for 10 min and then treated with ~60 mg (0.31 mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride. The pH was readjusted to 5.5–6.0 with $NaHCO_3$, and the suspension was shaken at room temperature for 24 h. The resin was collected by filtration, washed successively with 50 mL each of 0.1 M $NaHCO_3$ (pH 8), 1 mM HCl (pH 3), 0.5 M NaCl, and H_2O, and then stored at 4 °C in H_2O or in 20 mM Hepes buffer, pH 7.0, containing 1 mM dithiothreitol, 0.2 mM EDTA, 0.1 M KCl, and 10% (v/v) glycerol.

Standardization of Affinity Resins. The standardization of each affinity column involved a comparison of four samples: (a) the affinity resin itself (prepared as described above from 0.25 g of AH-Sepharose 4B and 11.6 μmol of ligand and dried by filtration on a fritted glass filter), (b) 0.25 g of AH-Sepharose 4B, (c) 1.36 μmol of the ligand employed for preparation of the affinity resin, and (d) a mixture of 0.25 g of AH-Sepharose 4B and 1.36 μmol of the same ligand. Each sample was suspended in 5 mL of 1 M HCl and heated at 100 °C for 15 min. The cooled solutions were each diluted to 50 mL with water and used for measurement of absorbance at 253 (for ligands **3**, **4**, and **8**) or 257 nm (for ligands **5**, **9**, **12**, **13**, **14**, **17**, and **18**).

The additivity of the ligand and resin absorptions was demonstrated by comparison of the sum of absorptivities obtained for samples b (0.51) and c (0.21 for ligand **5**) with that obtained for sample d (0.66 for **5**). As illustrated below for resin **7** (containing ligand **5**), the absorbance due to covalently bound ligand was determined by subtraction of the absorbance due to the resin from the sample absorbance (sample a – sample b; 0.67 A_{257} unit). The amount of ligand covalently bound to the resin was then calculated from this difference and from the absorptivity obtained with 1.36 μmol of ligand (sample c), i.e., (1.36 μmol/0.21 A_{257} unit)(0.67 A_{257} unit/0.25 g) = 17.4 μmol of ligand **5**/g of resin **7**, on the basis of the following (resin **7**; ligand **5**): (sample a) 0.250 g of resin **7**, 1.18 A_{257} units, (sample b) 0.250 g of AH-Sepharose 4B, 0.51 A_{257} unit, (sample c) 15 A_{257} units of ligand **5** (pH 5.0, 1.36 μmol), 0.21 A_{257} unit, and (sample d) 0.250 g of AH-Sepharose 4B and 15 A_{257} units of ligand **5** (pH 5.0, 1.36 μmol), 0.66 A_{257} unit. The results obtained with the other ligands are summarized in Table I.

Affinity Purification of Cap Binding Protein. Isolation of 24K CBP from preparations of rabbit reticulocyte protein synthesis initiation factors has been described (Sonenberg et al., 1979). Briefly, a 0–40% ammonium sulfate precipitate of the ribosomal high salt wash was sedimented in a 10–40% sucrose gradient containing 0.1 M KCl and buffer A (20 mM Hepes, pH 7.6, 1 mM dithiothreitol, 0.2 mM EDTA, and 10% glycerol). The upper half of the gradient was pooled to exclude high molecular weight initiation factor eIF-3. Pooled material was loaded directly onto the indicated resin (1 × 0.7 cm column). After the column was extensively washed with 50 mL of 0.1 M KCl in buffer A, it was eluted with 5 mL of 70 μM m^7GDP followed by 5 mL of 1 M KCl, both in the same buffer. Eluates were analyzed by electrophoresis in 10–18% polyacrylamide gels (Sonenberg et al., 1979).

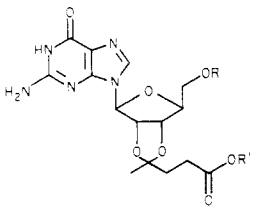
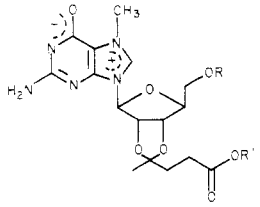
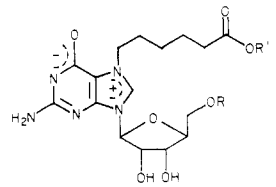
Assay for Cap Binding and Translation Stimulatory Activities. Fractions from the affinity column were tested for cap binding by cross-linking to periodate-oxidized, 5'-[^{3}H]-methyl-labeled reovirus mRNA (Sonenberg et al., 1978). Translation stimulatory activity of the affinity purified proteins was assayed in HeLa cell protein synthesizing extracts as described in detail previously (Sonenberg et al., 1980).

Results

The syntheses of affinity ligands **4** and **5** were accomplished starting from 2',3'-*O*-[1-(3-ethoxy-3-oxopropyl)ethylidene]-guanosine (**1**) (Seela & Waldek, 1975), which was converted to the respective ribonucleoside 5'-monophosphate (**2**) by treatment with pyrophosphoryl chloride in acetonitrile (Imai et al., 1969). Treatment of **2** with 1,1'-carbonyldiimidazole

[2] Ligand **18** was added in 2 mL of 50% aqueous dioxane.

Table I: Extent of Binding of Individual Ligands to AH-Sepharose 4B

ligand	compound no.	R	R′	extent of binding (μmol of ligand/g of resin)[a]
	4	$PO_3PO_3^{3-}$	Na^+	20.3
	8	H	Na^+	18.3
	3	$PO_3PO_3^{3-}$	Et	0
	5	$PO_3PO_3^{3-}$	Na^+	17.4
	9	H	Na^+	17.5
	12	$PO_3PO_3^{3-}$	Et	0
	13	$PO_3PO_3^{3-}$	Na^+	36.6
	14	H	Na^+	40.8
	17	$PO_3PO_3^{3-}$	CH_2CCl_3	2.0
	18	H	CH_2CCl_3	2.0

[a] Determined as indicated under Experimental Procedures.

afforded the nucleoside phosphorimidazolidate, which was not isolated but rather converted immediately to 2′,3′-*O*-[1-(3-ethoxy-3-oxopropyl)ethylidene]guanosine 5′-diphosphate (**3**) (Hoard & Ott, 1965; Kozarich et al., 1973). Although adventitious hydrolysis of the ester moieties in **1** and **2** was found to be a serious technical problem, the ethyl ester in **3** was found to be much more stable; successful saponification required the use of 0.5 M NaOH. Selective N-methylation of **4** (Adams et al., 1978) in anhydrous dimethyl sulfoxide (CH_3I, hydrogen chloride) then provided 2′,3′-*O*-[1-(2-carboxyethyl)-ethylidene]-7-methylguanosine 5′-diphosphate (**5**) in 40% overall yield from **1**. Affinity ligand **5** (and "control" ligand **4**) were coupled to AH-Sepharose 4B via the agency of a water-soluble carbodiimide. Also prepared as potential ligands, to permit verification of the mode of coupling of **4** and **5** to AH-Sepharose 4B, were 7-methylguanosine derivatives **9** and **12**.

Also prepared for study were affinity ligand **13** and the respective "control" ligand **14**. While the alkylation of guanosine and GDP with methyl iodide was found to be virtually complete within 4 h (Adams et al., 1978), the analogous alkylation of GDP with 6-iodohexanoic acid was <30% complete after 48 h, and the separation of **13** from GDP by ion-exchange chromatography was found to be difficult. A homogeneous sample of **13** was obtained by the use of a large excess of alkylating agent over a longer period of time. The corresponding ribonucleoside (**14**) was prepared in low yield by modification of the procedure of Jones & Robins (1963). Also prepared, by reaction of GDP and guanosine with 2,2,2-trichloroethyl 6-iodohexanoate, were 7-alkylguanosine derivatives **17** and **18**. 7-Alkylguanosine derivatives are known to have limited stability (Hecht et al., 1976), and all of the ligands prepared were stored frozen in sealed ampules at low temperature.

The amount of each ligand that was covalently bound to AH-Sepharose 4B was determined by the procedure of Seela & Waldek (1975). When prepared as described under Experimental Procedures, affinity resins **6** and **7** contained 20.3 and 17.4 μmol of ligand/g of Sepharose, respectively, while affinity resins **15** and **16** contained 36.6 and 40.8 μmol of ligand/g of Sepharose, respectively.

Since treatment of the guanosine derivatives prepared here with a carbodiimide could plausibly result in reactions involving the phosphate esters or 2-amino groups, it was necessary to show that attachment of the ligands to AH-Sepharose 4B had occurred only as intended, i.e., through the carboxylate moieties. Reaction of **4**, **5**, and **13** with *n*-pentylamine, under the conditions used for preparation of the affinity resins, resulted in each case in the formation of a new, less negatively charged product (30–40% yields), as judged by mobility on DEAE-cellulose and poly(ethylenimine). The ultraviolet spectra of each of the products was identical with that of the respective starting material, reflecting the absence of guanidine formation (Metz & Brown, 1969) between the carbodiimide and purine during coupling (Seela & Waldek, 1975; Rosemeyer & Seela, 1978).

That the ligands were coupled to Sepharose through the carboxylates was demonstrated by the series of experiments summarized in Table I. As shown, ligands **4** and **8**, differing only in the extent of modification of C-5′, were bound to the gel essentially equally as well. Very similar results were also obtained with ligands **5** and **9**, the N^7-methylated analogues of **4** and **8**. Thus the extent of ligand coupling to AH-Sepharose 4B was found to be independent of the presence of a 5′-diphosphate (and so presumably does not involve a phosphate group where these are present). On the other hand, the structurally related ligands lacking a free carboxylate (**3** and **12**) were found not to bind to the Sepharose at all, indicating that the carboxylate must represent the site of ligand attachment for ligands of this type. Analogously, ligands **13** and **14** were found to couple to AH-Sepharose 4B to essentially the same extent, although only the former contains a (di)-phosphate group. As anticipated, the structurally related ligands (**17** and **18**) lacking a free carboxylate could not be bound to the Sepharose to a significant extent.

Eukaryotic protein synthesis factor preparations contain a polypeptide of apparent molecular weight ~24 000 that binds to the 5′-terminal cap of mRNA (Sonenberg et al., 1978). It

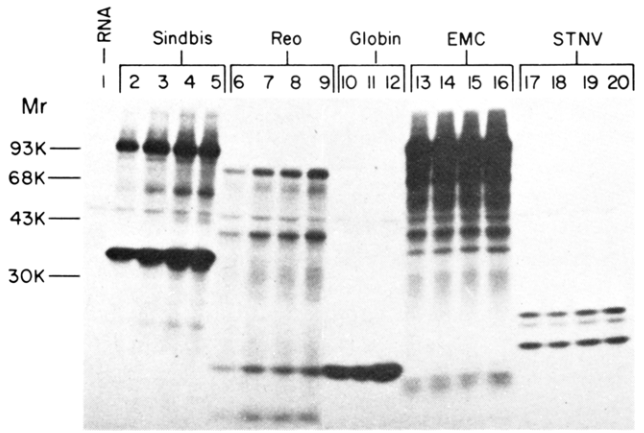


FIGURE 1: Analysis of cell-free products directed by capped and uncapped mRNAs. Hela cell-free protein synthesizing extracts were incubated for 60 min with [^{35}S]methionine and limiting amounts of Sindbis virus mRNA (~0.5 μg), reovirus mRNA (1.5 μg), rabbit globin mRNA (1.5 μg), EMC virus RNA (0.5 μg), or STNV RNA (1.5 μg). Cap binding protein was present in the reactions as follows: none, lanes 2, 6, 10, 13, and 17; ~15 ng; lanes 3, 7, 11, 14, and 18; ~30 ng, lanes 4, 8, 12, 15, and 19; ~45 ng, lanes 5, 9, 16, and 20. (Lane 1) No RNA or cap binding protein added. Products were analyzed by polyacrylamide gel electrophoresis and autoradiography as described previously (Sonenberg et al., 1980).

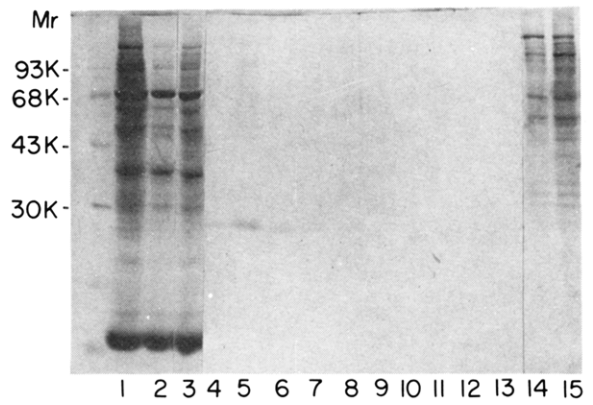


FIGURE 2: Stained gel profiles of protein fractions from affinity columns. A total of 38 mg each of eukaryotic initiation factors partially purified by ammonium sulfate precipitation and sucrose gradient centrifugation was applied to columns (1 × 0.7 cm) of 7-carboxypentyl-GDP–Sepharose and 7-carboxypentyl-G–Sepharose in 0.1 M KCl and buffer A as described under Experimental Procedures. After the columns were washed in the same buffer, they were eluted with 5 mL of 70 μM m^{7}GDP (five 1-mL fractions) followed by 1 M KCl. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue (Sonenberg et al., 1979). (Left-hand lane) Marker proteins: rabbit muscle phosphorylase *a* (M_r 93 K), bovine serum albumin (68K), ovalbumin (43K), and carbonic anhydrase (30K); (lane 1) 76 μg of the loaded protein; (lane 2) 22 μg of 26 mg total flow-through protein from 7-carboxypentyl-GDP–resin; (lane 3) 28 μg of 28 mg of total flow-through protein from 7-carboxypentyl-G–resin; (lanes 4–8) 30 μL each of the 1-mL m^{7}GDP eluates from 7-carboxypentyl-GDP–resin (total 24K CBP recovered ~30 μg); (lanes 9–13) 30 μL of each m^{7}GDP eluate fraction from 7-carboxypentyl-G–resin; (lane 14) 8 μg of 7 mg of protein recovered from 7-carboxypentyl-GDP–resin in 1 M KCl fraction; (lane 15) 12 μg of 8 mg of protein from 7-carboxypentyl-G–resin in 1 M KCl fraction.

was characterized as a "cap binding protein" (24K CBP) on the basis of specific, i.e., m^7 GDP-inhibited, cross-linking to the capped end of periodate-oxidized mRNAs (Sonenberg et al., 1978). Because the 24K CBP was found associated with two different initiation factors, eIF-3 and eIF-4B, it was necessary to develop a CBP purification scheme before functional studies could be carried out.

The experimental approach that proved particularly effective was affinity chromatography on resins prepared and characterized as detailed above. A previous preliminary report described the purification of 24K CBP by adsorption to m^{7}GDP–Sepharose and elution in the presence of the cap analogue m^{7}GDP (Sonenberg et al., 1979). In contrast to essentially quantitative binding to the m^{7}GDP resin, only a small fraction of 24K CBP was retained by GDP–Sepharose. Affinity-purified 24K CBP was functionally active and differentially stimulated the translation of capped mRNAs relative to naturally uncapped messengers in HeLa cell extracts (Sonenberg et al., 1980). Stimulation was obtained by addition of a low level of 24K CBP (15–45 ng/25 μL) to extracts directed by capped mRNAs of viral (Sindbis, reovirus) or cellular (globin) origin, and the increase in [^{35}S]methionine-labeled products was generally greater at higher concentrations of added CBP (Figure 1, lanes 1–12). Translation of uncapped RNAs from mouse encephalomyocarditis (EMC) virus and satellite tobacco necrosis virus (STNV) was essentially unaffected by addition of 24K CBP (Figure 1, lanes 13–20).

These results demonstrate the usefulness as affinity substrates of guanosine nucleotides coupled to Sepharose via a modified ribose moiety. Because 7-ethyl-GDP and 7-benzyl-GDP inhibited capped mRNA binding to ribosomes as effectively as the "normal" cap analogue, m^{7}GDP (Adams et al., 1978), it seemed likely that 7-substituted GDP derivatives coupled to Sepharose via the 7-substituent would also provide resins with affinity for cap binding proteins. Columns of Sepharose-coupled 7-carboxypentyl-GDP and 7-carboxypentyl-G were prepared as described, and their affinity properties compared. Initiation factor preparations, partially purified by ammonium sulfate precipitation and sucrose gradient sedimentation, were loaded onto the columns as described under Experimental Procedures. Aliquots of the flow-through material, the m^{7}GDP eluates, and the 1 M KCl wash fractions were analyzed by polyacrylamide gel electrophoresis (Figure 2). The stained gel profiles indicated that a M_r 24 000 polypeptide (putative 24K CBP) was specifically eluted from the 7-carboxypentyl-GDP–Sepharose column by 70 μM m^{7}GDP (Figure 2, lanes 4–8). No protein band was detected in the corresponding fractions from the 7-carboxypentyl-G–Sepharose (Figure 2, lanes 9–13).

From the gel profiles in Figure 2, it is clear that 7-carboxypentyl-GDP–Sepharose, but not the nucleoside derivative, is a potentially useful affinity substrate for purifying cap binding proteins. However, the resin-coupled 7-carboxypentyl-GDP was less satisfactory for this purpose than m^{7}GDP–Sepharose under the conditions employed. This became evident when the various column fractions were tested for cap binding protein activity by cross-linking to oxidized, 5′-radiolabeled reovirus mRNA. As shown in the fluorogram in Figure 3, the 24K CBP in the applied sample (lane 1) bound to both Sepharose-coupled 7-carboxypentyl-G and 7-carboxypentyl-GDP in the presence of 0.1 M KCl and was not apparent in the flow-through material (Figure 3, lanes 2 and 3). The radiolabeled bands of M_r ~50 000 in the flow-through fractions probably correspond to elongation factor 1 as described previously (Sonenberg et al., 1978, 1979). Elution of the 7-carboxypentyl-GDP–Sepharose with the cap analogue m^{7}GDP released some active 24K CBP (Figure 3, lanes 8 and 9), but the bulk of the 24K CBP was recovered in the 1 M KCl eluate (Figure 3, lane 5) together with many other proteins that did not cross-link to caps (Figure 2, lane 15). (Note the different exposure times for fluorograms shown in Figure 3; see legend.) In the case of the 7-carboxypentyl nucleoside column, 24K CBP was present in the 1 M KCl eluate (Figure 3, lane 4) but there was apparently none released by treatment

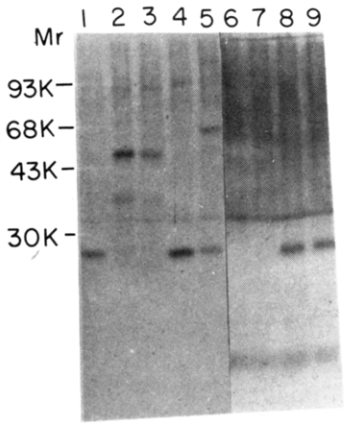


FIGURE 3: Cross-linking patterns of column protein fractions. Reovirus mRNA containing 5′-terminal, methyl-^{3}H-labeled m^7GpppG^m was synthesized and oxidized by periodate treatment as described (Sonenberg et al., 1978). Protein samples obtained in the various column fractions were bound and cross-linked to the mRNA and analyzed by gel electrophoresis as described in detail (Sonenberg et al., 1979). (Lane 1) 76 μg of 38 mg of total loaded protein; (lane 2) 56 μg of flow-through from 7-carboxypentyl-G–resin; (lane 3) 44 μg of flow-through from 7-carboxypentyl-GDP–resin; (lane 4) 8 μg of 1 M KCl eluate from nucleoside column; (lane 5) 8 μg of 1 M KCl eluate from nucleotide column; (lanes 6 and 7) 15 μL each of 1-mL m^7GDP eluate fractions 2 and 3 from 7-carboxypentyl-G–resin; (lanes 8 and 9) 15 μL each of 1-mL m^7GDP eluate fractions 2 and 3 from 7-carboxypentyl-GDP column. Fluorogram exposures were 14 days for lanes 1–5 and 53 days for lanes 6–9.

with m^7GDP (Figure 3, lanes 6 and 7). Furthermore, it is clear from a comparison of the stained and radiolabeled protein patterns of the same column fraction that the extent of crosslinking is not a direct indication of the amount of 24K-CBP (for example compare lanes 5 and 6 of Figure 2 with lanes 8 and 9 of Figure 3). It is not known if the cross-linking activity is diminished by removal of other proteins or if the 24K CBP retains a low level of tightly bound m^7GDP that inhibits cross-linking.

Discussion

Adams et al. (1978) have reported the preparation of a number of 7-alkylguanosine 5′-diphosphate derivatives which were tested for their ability to inhibit the formation of an initiation complex between (capped) reovirus mRNA and wheat germ ribosomes. Most structural alterations in the purine nucleus diminished the efficiency of the nucleotides as inhibitors of initiation complex formation, an observation that probably reflects structural requirements for cap recognition. Interestingly, 7-methylguanosine 5′-diphosphate derivatives modified in the carbohydrate moiety were fully as inhibitory as m^7GDP itself, consistent with the observation that mRNA cap recognition was not abolished by chemical modification of the carbohydrate in the m^7G cap (Sonenberg et al., 1978). Additionally, it was found that 7-ethyl- and 7-benzylguanosine 5′-diphosphates were essentially as inhibitory to initiation complex formation as 7-methylguanosine, suggesting that the size of the 7-substituent was not involved in cap recognition. Consistent with this suggestion was the subsequent finding that reovirus mRNA synthesized in the presence of *S*-adenosylethionine and consequently containing a 5′-terminal 7-ethyl-G cap was translated in vitro essentially as well as the corresponding m^7G-capped mRNA (Furuichi et al., 1979).

These findings have obvious implications for the design of cap analogues that may be employed to study the nature of cap recognition or as affinity ligands for the isolation of cap binding protein(s). In the latter case, e.g., the available data suggest that immobilization of the affinity ligand via the carbohydrate moiety or 7-substituent would probably interfere least with the affinity of the ligand for the relevant protein(s). On this basis, affinity ligands **5** and **13** were chosen for preparation. The synthesis of the requisite ligands and their immobilization on AH-Sepharose 4B (to give affinity resins **7** and **15**, respectively) are described above, as is the structural characterization of the resins. Also described is the preparation of resins **6** and **16**, designed to verify the specific nature of the affinity between individual proteins and resins **7** or **15**, respectively.

As observed for resin **7**, resin **15** also effectively bound 24K CBP. Elution with m^7GDP released the protein in active form, as determined by the cross-linking assay (Figure 3) and by stimulation of capped Sindbis virus mRNA translation in HeLa cell extracts (data not shown). However, cap analogue mediated release of 24K CBP from the resin was incomplete, as indicated by the high amount of cross-linking activity in the 1 M KCl eluate. It is possible that the yield of purified 24K CBP from resin **15** could be improved by using higher concentrations of m^7GDP and/or by elution with cap analogue in the presence of 0.1–1 M KCl. In contrast to resin **6** which was found to have a low capacity for retention of 24K CBP (Sonenberg et al., 1979), resin **16** apparently retained 24K CBP essentially completely. Binding appeared to be nonspecific because there was no elution from the column by 70 μM m^7GDP (Figure 2), although 24K CBP that was active by cross-linking was released from resin **16** by 1 M KCl (Figure 3). From these findings, it is evident that 7-substituted guanosine nucleotides coupled to Sepharose by either the ribose moiety or the 7-substituent provide a useful experimental approach for the isolation and characterization of proteins that interact with the m^7G cap structure found at the 5′ ends of the most eukaryotic mRNAs and their precursors. Application of similar affinity resins may allow detection of proteins that interact with the 2,2,7-trimethyl-G caps on low molecular weight nuclear RNAs (Reddy et al., 1974), molecules that may be involved in mRNA splicing (Lerner et al., 1980; Rogers & Wall, 1980). Affinity chromatography should also provide a basis for isolating cap binding protein(s) recently implicated in the priming of influenza virion associated RNA polymerase by capped heterologous mRNAs (Krug et al., 1980).

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Modulation of Tropoelastin Production and Elastin Messenger Ribonucleic Acid Activity in Developing Sheep Lung†

Shigeki Shibahara,* Jeffrey M. Davidson,‡ Kent Smith, and Ronald G. Crystal

ABSTRACT: During fetal development of the sheep lung, elastin content continually increases. For examination of the processes controlling this elastin accumulation, an explant culture system was characterized with respect to changes in tropoelastin production in sheep lung during fetal and early postnatal development. Relative tropoelastin production in cultured lung explants, quantitated by immunoprecipitation, was about 0.3% of total [^{14}C]valine incorporation during the period from 55 to 104 days after conception. This percentage began to increase by 112 days after conception, reached a maximum value of about 1.0% by 135 days after conception, and then declined to 0.5% soon after birth. The absolute rate of tropoelastin production paralleled these changes in relative tropoelastin production. For evaluation of the processes controlling tropoelastin production in the developing sheep lung, total cellular RNA prepared from 68-day-old fetal, 107-day-old fetal, and 147-day-old fetal lung was translated in a rabbit reticulocyte lysate system. Elastin mRNA activity, expressed as the amount of elastin precursor translated per microgram of DNA, increased about 3-fold during fetal lung development, and elastin precursor synthesis, expressed as a proportion of total translational activity, increased in parallel. It appears, therefore, that elastin production in developing fetal lung is modulated, at least in part, by the amount of available translatable elastin mRNA present in the tissue.

During fetal life, the lung undergoes marked morphological changes to become a stable gas-exchange system at birth. Beyond the very early embryonic stages of morphogenesis, three prenatal stages of lung development are traditionally recognized: the glandular, canalicular, and alveolar or saccular stages (Emery, 1969; Meyrick & Reid, 1977). Morphological development continues after birth and through infancy (Loosli & Potter, 1959; Boyden & Tompsett, 1965).

Connective tissue elements appear to play a major role in lung development (Emery, 1969). One of these elements, elastin, is the component of elastic fibers which possesses the elastic properties critical to the mechanical behavior of the lung as a gas-exchanging organ. Morphologic studies of fetal lung of a variety of species have indicated marked age-related changes in the amount and distribution of elastic fibers (Loosli & Potter, 1959; Jones & Barson, 1971; Collet & Des Biens, 1974) that are thought to be essential to the proper architectural development of lung.

In this context, the present study was designed to evaluate how the lung regulates elastin production during fetal life and the early perinatal period. Three aspects of lung elastin were evaluated in the developing sheep: (1) the amount of elastin present, (2) the production of tropoelastin[1] by lung explants, and (3) the role of elastin mRNA levels in regulating elastin synthesis during lung development. To accomplish these objectives, we developed in vitro systems for quantitating the amount of tropoelastin production by lung and corresponding levels of lung elastin mRNA activity.

Materials and Methods

Materials. All reagents were of highest commercial grade available. Culture media were prepared by the NIH Media Unit. Killed *Staphylococcus aureus* cells ("Pansorbin") were from Calbiochem (La Jolla, CA), L-[2,3,4-^{3}H]valine (11.1 Ci/mmol) was from New England Nuclear (Boston, MA), and L-[^{14}C]valine (250 mCi/mmol) was from Schwarz/Mann (Orangeburg, NY).

Solutions. The "extraction" solution used to extract labeled proteins from lung explants was a mixture of 5% deoxycholate, 5% Triton X-100, 70 mM *N*-ethylmaleimide, 175 μg/mL toluenesulfonylphenylalanyl chloromethyl ketone, 175 μg/mL

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[1] The term "tropoelastin" is used to refer to the soluble elastin precursor present in tissue extracts and culture media of lung explants. The term "elastin precursor" designates the primary translation product of elastin mRNA.