Solubilized DNA-Dependent RNA Polymerase Activities in Resting and Growing Fibroblast†

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ABSTRACT: The activities of RNA polymerases I and II have been measured in 3T6 during the transition from the resting to growing state by solubilization of the enzymes followed by chromatography on DEAE-Sephadex columns. The activity of RNA polymerase II remains unchanged during the first 12 h after serum stimulation while the activity of RNA polymerase I increases and closely parallels the increased activity seen in isolated nuclei. Compared to enzyme from resting cells, RNA polymerase I from serum stimulated cells elutes at a lower ammonium sulfate concentration on DEAE-Sephadex chromatography and its activity shows distinctly different dependencies on the concentration of ammonium sulfate and magnesium ion. These observations are discussed in relation to the possible mechanism by which 3T6 regulates the synthesis of preribosomal RNA.

Addition of calf serum, which contains fibroblast growth factors, to a resting culture of 3T6 stimulates a transition to the growing state. When the rates of RNA synthesis are measured in isolated nuclei there is an increase in the rate of preribosomal RNA synthesis as early as 10 min after the addition of serum. The rate doubles in about 5 h and is three- to fourfold greater by the time the cells begin to synthesize DNA 12 h later (Mauck and Green, 1973). The rate of synthesis of Hn-RNA[1] does not change during the first 12 h following serum stimulation and the rate of synthesis of pretransfer RNA increases approximately 1.5-fold (Mauck and Green, 1974). Similar changes in rates of RNA synthesis during the transition from a resting to growing state have been observed in WI38 (Bombik and Baserga, 1974) and in CHO cells grown in suspension culture (McReynolds, 1974). The mechanism(s) by which the cellular RNA polymerases respond so rapidly to serum growth factors has not been established in any of these cell lines. Increased activity of the RNA polymerases could result from an increased concentration of enzyme, a modification of the template, a change in the polymerase molecule itself, or a combination of these factors.

Eukaryotic cells have been shown to contain at least four distinct species of DNA-dependent RNA polymerase (Schwartz et al., 1974). The presence of multiple forms of RNA polymerase could allow the cell to independently regulate the cellular levels of the various RNA species. The levels of DNA-dependent RNA polymerase have been measured in a number of cell types with widely varying growth rates and it has been suggested that the increased activity seen in more rapidly growing cells is due to an increased number of enzyme molecules, with RNA polymerase I showing the greatest variability in activity (Roeder et al., 1975).

A comparison of the activities of solubilized and partially purified enzymes on exogenous substrate allows one to distinguish between changes in enzymatic activity due to template modification or changes in either enzyme concentration (Weinman et al., 1976) or catalytic efficiency. The studies reported below describe the solubilization, chromatographic separation, and measurement of RNA polymerase I and II activities in 3T6 before and after the cells were stimulated to enter a division cycle.

Experimental Procedures

Cell Culture and Isolation. The established mouse fibroblast cell line 3T6 was obtained from Dr. Howard Green of the Massachusetts Institute of Technology and was grown in the Dulbecco Voget modification of Eagle's medium supplemented with 10% calf serum. Resting cultures of 3T6 were prepared by plating 3.5×10^6 cells on 100-mm petri dishes in medium containing 0.5% calf serum. The medium was changed on the second and fourth days and the cells were ready to use on the seventh day when fewer than 0.5% of the cells were synthesizing DNA. To initiate the transition from the resting state, the medium was removed and medium, prewarmed to 37 °C, containing 10% calf serum was added to the cells. Addition of calf serum to 10% gave identical results (data not shown).

To harvest resting cells, the medium was removed, 5 mL of ice-cold medium containing 0.5% calf serum and 0.025 M Hepes (pH 7.0, 0 °C) was added, and the cells were scraped from the growth vessel with a rubber policeman. Cells stimulated to grow were harvested in a similar manner except that the harvest medium contained 10% calf serum. The cells were centrifuged at 2000 rpm for 5 min and the medium removed. The cells were either used immediately for enzyme solubilization or the cell pellet was frozen in a dry ice–acetone bath and stored at −76 °C for later use.

Enzyme Solubilization. The method used for enzyme solubilization was essentially that of Jaehning et al. (1975). Cell pellets were brought to a final volume of 1.5 mL in buffer A (0.05 M Tris-Cl (pH 7.9, 25 °C), 25% glycerol (v/v), 0.1 mM EDTA, 1 mg/ml bovine serum albumin, 0.5 mM dithiothreitol). Ammonium sulfate (3 M; pH 7.9) was added to give a final concentration of 0.30 M and the solution was sonicated 3 times for 10 s with a Branson sonicator (microprobe, setting 3) at 0–4 °C. The resulting suspension (FIA) was centrifuged at 20 000 rpm for 5 h. The supernatant (FIB) was diluted to 0.10 M ammonium sulfate with buffer A and centrifuged at 40 000 rpm for 90 min. The supernatant (F-2)

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[1] Abbreviations used are: Hn-RNA, heterogeneous nuclear ribonucleic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; UMP and UTP, uridine mono- and triphosphate.

TABLE I: Recovery of RNA Polymerase Activity after Solubilization and DEAE-Sephadex Chromatography (Units of RNA Polymerase/3.5 × 10^7 Cells).[a]

Fraction	Resting I + III[b]	Resting II	Recovery %	6 h after Serum Stimulation I + III[a]	6 h after Serum Stimulation II	Recovery (%)
FIA	96	76	100	133	71	100
FIB	101	137	138	171	149	157
F2	148	71	127	174	45	107
DEAE-Sephadex	98 (I)	215	181	199 (I)	234	212

[a] 3.5 × 10^7 resting cells and 3.5 × 10^7 cells stimulated for 6 h with medium containing 10% calf serum were subjected to the enzyme solubilization and chromatographic procedures outlined under Experimental Procedures. Assays of fraction FIA, FIB, and F-2 were performed at 0.05 M ammonium sulfate. Column fractions were assayed at the ammonium sulfate concentration resulting from dilution of 20 μl of each fraction to a final volume of 50 μL. RNA polymerase I + III activities present in FIA, FIB, and F-2 are those resistant to 1 μg/mL α-amanitin while units of II represent activity sensitivity to 1 μg/mL α-amanitin. The chromatographic profiles of these preparations are shown in Figure 1. The units of activity of I and II were calculated from the individual peaks of activity. Calf-thymus DNA was used as template in all the experiments reported in this paper. [b] Prior to chromatography on DEAE-cellulose, the activities measured in the presence of α-amanitin represent RNA polymerases I and III. For the reasons outlined in the text, only RNA polymerases I and II were measured from the data obtained from DEAE-Sephadex chromatography.

was diluted to 0.05 M ammonium sulfate with buffer A and chromatographed as described below.

Chromatography. DEAE-Sephadex (A-25) chromatography was carried out as described by Schwartz et al. (1974). Protein was loaded at a concentration of ≤2 mg/ml bed volume and the enzymes were eluted with a 3 column volume linear gradient from 0.05 to 0.50 ammonium sulfate in buffer A at the rate of 0.42 ml/min. Individual column conditions are listed in the figures.

RNA Polymerase Assay. The assay was carried out as described by Schwartz et al. (1974) except that, when used, α-amanitin was present at 1 μg/mL; the reaction mixture was 5 μM in cold UTP and contained 1.5 μCi of [^{3}H]UTP (sp act. 35–50 Ci/mmol). Calf-thymus DNA was purchased from Sigma and the same preparation was used throughout these experiments. Peak activity tubes from each time point gave a linear reaction during the 20-min incubation period and the velocity of the reaction was proportional to the amount of enzyme added (data not shown). To obtain the number of units of enzyme at saturating levels of UTP, the numbers presented should be multiplied by 3.

[^{3}H]UMP bound to DEAE-81 filter paper is counted less efficiently than free [^{3}H]UMP. A series of duplicate assays was run utilizing RNA polymerase I obtained by DEAE-Sephadex chromatography. Half of the filters were oxidized in a Packard sample oxidizer and the resulting ^3H_2O was counted under identical conditions with the remaining filters. The ratio thus obtained was used to correct for the difference in counting efficiencies.

Miscellaneous Procedures. Protein concentrations were measured by the method of Mauck (1976), and ammonium sulfate concentrations were measured with a Radiometer conductivity meter after a 200-fold dilution of the sample.

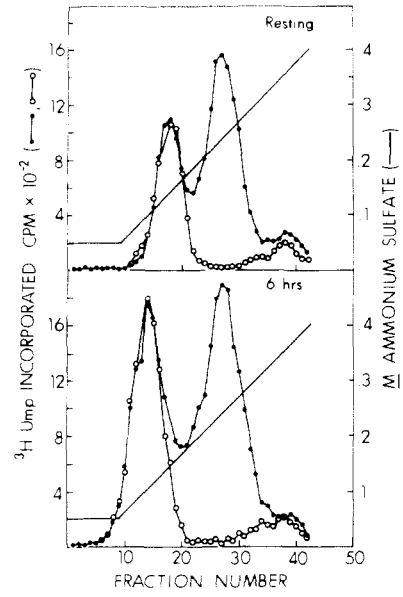

FIGURE 1: DEAE-Sephadex chromatography of the solubilized RNA polymerases from resting cells and serum stimulated cells. Fraction F-2 from resting cells contained 15.2 mg of protein and F-2 from cells stimulated for 6 h contained 18 mg of protein. Both were chromatographed on 9-mL DEAE-Sephadex columns. Fractions (0.84 mL) were collected from a 27-mL gradient: activity in the absence (●) or presence (○) of 1 μg/mL α-amanitin.

Results

Solubilization and DEAE-Sephadex Chromatography of Solubilized RNA Polymerases. The RNA polymerases present in resting cells and cells stimulated for 6 h were solubilized and chromatographed as described under Experimental Procedures. Table I shows the recovery of activity at each step. There is considerable fluctuation in the activities during the centrifugation steps. This has been seen by others and may be due to the removal of inhibitors during centrifugation or the presence of variable quantities of DNA which make the enzymes less than totally dependent on exogenous substrate (Weinman et al., 1976). The activities recovered following DEAE-Sephadex chromatography vary little from preparation to preparation and the data in Table I indicate there is no detectable loss of activity during the isolation of the enzymes.

The chromatographic profile of the enzymes from resting cells and cells stimulated for 6 h is shown in Figure 1. The first activity peak elutes at about 0.15–0.16 M ammonium sulfate in resting cells and at about 0.11 M ammonium sulfate in cells stimulated for 6 h. The activity in the presence of 1 μg/ml α-amanitin is the same as in its absence. The second activity peak elutes at about 0.24–0.26 M ammonium sulfate in both resting cells and serum stimulated cells and is completely inhibited by 1 μg/ml α-amanitin. In the presence of 1 μg/ml α-amanitin two additional peaks are seen at about 0.30 and 0.35 M ammonium sulfate.

The first peak has been classified as RNA polymerase I due to its elution position, insensitivity to 200 μg/ml α-amanitin (data not shown), and characteristic Mg^{2+}, Mn^{2+}, and $(NH_4)_2SO_4$ activation profiles (Roeder, 1974). The second peak has been classified as RNA polymerase II due to its elution position and sensitivity to low concentration of α-amanitin (Lindell et al., 1970). The two additional peaks which elute at higher ammonium sulfate concentrations and are detected when enzyme II is inhibited with α-amanitin are tentatively identified as class III enzymes (Weil and Blatti, 1976).

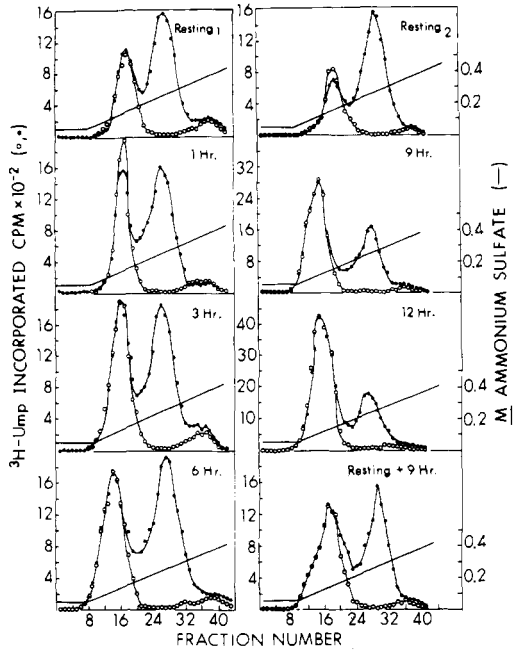

FIGURE 2: DEAE-Sephadex chromatography of RNA polymerase activities isolated at various times following serum stimulation. The F-2 fraction obtained from resting cells (two separate experiments) and at 1, 3, 6, 9, and 12 h following serum stimulation contained 14.1, 15.2, 18.0, 15.1, 18.3, and 20.3 mg of protein, respectively. 1.75×10^6 cells stimulated for 9 h and 1.40×10^6 resting cells were mixed at harvest. The F-2 fraction contained 15.0 mg of protein. The two resting cell samples, the mixed cell sample, and the 1-, 3-, and 6-h samples were chromatographed on 9-mL DEAE-Sephadex columns. Fractions of 0.84 mL were collected from a 27-mL gradient. The 9- and 12-h samples were chromatographed on 10-mL DEAE-Sephadex columns and eluted with a 30-mL gradient; 0.92-mL fractions were collected. Aliquots of 20 μL were assayed in the absence (●) or presence (○) of 1 μg/mL α-amanitin. Please note that the ammonium sulfate gradient profiles are not identical in each case.

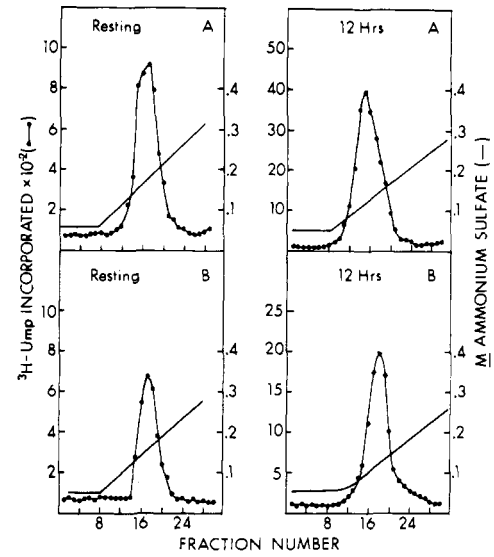

FIGURE 3: Rechromatography of RNA polymerase I from resting and serum stimulated cells. Enzyme from 3.5×10^7 resting cells (16 mg of protein) and 3.5×10^7 cells stimulated for 12 h (20.1 mg of protein) was chromatographed on 10-ml DEAE-Sephadex columns (A). Fractions of 0.92 mL were collected from a 30-mL gradient at 0.42 mL/min. Fractions 14–22 from the resting cells were pooled, diluted to 0.05 M ammonium sulfate with buffer A, and rechromatographed under identical conditions (B). Approximately 70% of the pooled activity was recovered from the second column. RNA polymerase I from serum stimulated cells is much more sensitive to dilution than is the enzyme from resting cells. To minimize this effect, fraction 15 was diluted onefold with buffer A and rechromatographed on a 2-mL DEAE-Sephadex column. Fractions of 0.18 mL were collected from a 6-mL gradient at the rate of 0.10 mL/min. The first 30 fractions of each column were assayed in the presence of 1 μg/mL α-amanitin.

However, the low levels of activity which are detected under these assay conditions preclude quantitative measurement and any further characterization. Removal of the DNA from the high salt sonicate by absorption to DEAE-cellulose (Weinman et al., 1976) gave no increase in yield of these enzymes. Utilization of poly[d(A-T)] as template gave about a twofold increase in activity over calf-thymus DNA (Weinman et al., 1976), but it is felt that the data obtained for the class III enzymes are not sufficiently accurate to permit any conclusion regarding changes in their activity following serum stimulation. For this reason only RNA polymerase I and II activities are reported in the data presented below.

Solubilized RNA Polymerase Activities during the Transition from Resting to Growing State. Figure 2 shows the DEAE-Sephadex profiles of the RNA polymerases isolated from cells harvested at different times following serum stimulation. Following serum stimulation there is an increase in the activity of RNA polymerase I and an increasing tendency for the enzyme to elute at a lower ammonium sulfate concentration. Three hours after serum stimulation the enzyme elutes at about 0.14 M ammonium sulfate when compared to the resting cell enzyme which consistently elutes at 0.15 to 0.16 M ammonium sulfate. By 6 h the enzyme elutes between 0.10 and 0.11 M ammonium sulfate and continues to elute at this concentration at 9 and 12 h after serum stimulation. Rechromatography of RNA polymerase I on DEAE-Sephadex from resting or serum stimulated cells gives the same profile (Figure 3). RNA polymerase II elutes at about 0.24–0.26 M ammonium sulfate at all time points examined. If resting cells and

TABLE II: RNA Polymerase Activities in 3T6 following Serum Addition.[a]

Time after Serum Stimulation (h)	Units/3.5×10^7 Cells		Rel Increase	
	I	II	I	II
Resting	98	215	1.00	1.00
1	126	180	1.28	0.84
3	172	206	1.76	0.96
6_1[b]	199	234	2.03	1.09
6_2[c]	217	195	2.20	0.91
9	263	162	2.68	0.75
12	394	213	4.02	0.99

[a] The enzyme activities were calculated from the data presented in Figure 2. Each point represents the activity from 3.5×10^7 cells. Enzyme III was not quantitated for the reasons outlined in the text. [b,c] Two separate experiments.

cells stimulated for 9 h are mixed at harvest and then fractionated, the RNA polymerase I activity peak broadens and elutes at an intermediate ammonium sulfate concentration of 0.14 M while RNA polymerase II activity still elutes at about 0.25 M ammonium sulfate.

The number of units of RNA polymerases I and II was determined from the data presented in Figure 2 and these data are presented in Table II. The levels of RNA polymerase II are relatively constant during the first 12 h following serum stimulation. However, the activity of RNA polymerase I increases and closely parallels the activity seen in isolated nuclei following serum stimulation (Mauck and Green, 1973).

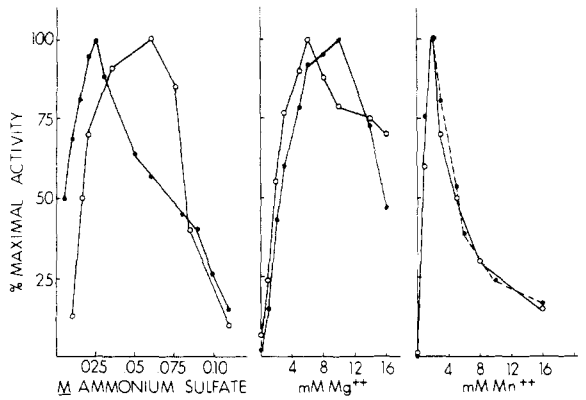

FIGURE 4: Effect of ammonium sulfate, Mg^{2+}, and Mn^{2+} on RNA polymerase I from resting and serum stimulated cells. Enzyme was obtained from DEAE-Sephadex chromatography and dialyzed against buffer A without bovine serum albumin containing 0.03 M ammonium sulfate. Aliquots were then assayed under standard conditions at the salt and metal ion concentrations indicated. The Mg^{2+} and Mn^{2+} curves were assayed in 0.05 M $(NH_4)_2SO_4$ for both enzymes: (●) RNA polymerase I from cells stimulated for 6 h; (○) RNA polymerase I from resting cells.

Ammonium Sulfate, Mg^{2+}, and Mn^{2+} Activation Profiles. RNA polymerase I was isolated by DEAE-Sephadex chromatography from resting cells and cells stimulated for 6 h. The ammonium sulfate, Mg^{2+}, and Mn^{2+} activity profiles for these enzymes are shown in Figure 4. RNA polymerase I isolated from resting cells shows a rather broad ammonium sulfate activity profile and is optimally active at 0.06 M while the enzyme from serum stimulated cells demonstrated a rather sharp peak of activity at 0.02 M ammonium sulfate. Both enzymes show a sharp peak of activity at 2 mM Mn^{2+}. The resting cell enzyme shows a peak activity at 6 mM Mg^{2+} while the enzyme from stimulated cells enzyme has maximal activity at 10 mM Mg^{2+}. Both enzymes are about 1.3 times as active with Mn^{2+} than Mg^{2+} at optimal concentrations.

Discussion

The rate of RNA synthesis in the mouse-fibroblast line 3T6 can be measured as the incorporation of labeled ribonucleoside triphosphates by nonviable "ghost monolayers" prepared with the aid of the detergent NP-40 (Tsai and Green, 1973). Estimates of the rate of RNA synthesis under these conditions are not affected by the cell-membrane permeability barrier and the slowly equilibrating cell nucleotide pools, which complicate interpretation of incorporation rates in whole cells. After stimulation of resting cultures of 3T6 with serum containing fibroblast growth factors, an increase in total RNA synthesis could be detected in ghost monolayers prepared less than 30 min later (Tsai and Green, 1973). Further investigations showed that the entrance of resting cells into a division cycle is accompanied by very different changes in the synthesis of pre-rRNA, heterogeneous nuclear RNA, and pre-transfer RNA (Mauck and Green, 1973, 1974). Following serum stimulation of resting 3T6 cells, there is an increase in the rate of pre-rRNA synthesis (measured in ghost monolayers) within 10 min, or about 1% of a generation time.

Changes in the activity of RNA polymerase I have been observed in rat liver nuclei following hydrocortisone administration (Sajdel and Jacob, 1971), treatment of seedlings with the auxin 2,4-dichlorophenoxyacetic acid (Guilfoyle et al., 1975), treatment of resting human lymphocytes with phytohemagglutinin (Jaehning et al., 1975), secondary stimulation of chick oviduct cells with estradiol (Cox, 1976), Ehrlich ascites tumor cells following inhibition of protein synthesis (Grummt et al., 1976), and in human KB cells following infection with adenovirus 2 (Weinman et al., 1976). Several mechanisms have been postulated to account for these changes in the activity of RNA polymerase I. These include an increased number of RNA polymerase molecules (Cox, 1976), differences in the amount of "free" vs. bound RNA polymerase (Grummt et al., 1976), changes in the template (Farber et al., 1972), changes in the intracellular pool size of ATP and GTP (Grummt and Grummt, 1976), and alteration of preexisting RNA polymerase I molecules (Guilfoyle et al., 1975; Sajdel and Jacob, 1971). The studies reported here were undertaken in order to establish whether the increased activity of RNA polymerase I seen in 3T6 following serum stimulation is due to alterations in the template or changes in enzyme concentration and/or catalytic efficiency (Weinman et al., 1976).

The data show that in 3T6, the activity of the solubilized RNA polymerase II remains constant during the first 12 h following serum stimulation, which is in agreement with the data obtained utilizing isolated nuclei (Mauck and Green, 1973). However, the RNA polymerase I activity increases and closely parallels the activity seen in isolated nuclei. The present studies do not allow one to distinguish between increased enzyme concentration or a change in the catalytic efficiency of preexisting enzyme as being responsible for the observed increases in activity of RNA polymerase I. A mechanism depending on an increase in the synthesis of a specific protein, itself depending on an increase in the synthesis of its mRNA, is probably excluded since the synthesis of mRNA and its transport to the cytoplasam require at least 15–20 min (Penman et al., 1968). If a change in the rate of synthesis of a specific protein is involved, this change would have to be effected at the level of translation.

The partially purified RNA polymerase I from serum stimulated cells elutes at a lower $(NH_4)_2SO_4$ concentration on DEAE-Sephadex and has apparently different ammonium sulfate and magnesium ion sensitivities when compared to enzyme from the resting cell. These differences suggest that the enzyme from serum stimulated cells might be different from that present in resting cells. However, at present, the enzymes are not pure enough to rule out the possibility of contaminating proteins being responsible for these differences. Further purification of RNA polymerase I from resting and serum stimulated cells is currently underway in order to determine if the increased activity is due to changes in the concentration of enzyme and/or a change in its catalytic efficiency.

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Complete Nucleotide Sequence and Properties of the Major Species of Glycine Transfer RNA from Wheat Germ†

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ABSTRACT: The complete nucleotide sequence of wheat germ $tRNA_1^{Gly}$ has been determined. It is a small tRNA, containing only 74 nucleotides of which 10 are methylated or otherwise modified. Several important features of this tRNA are: (a) a complete absence of ribothymidine which is the most common methylated nucleoside in tRNA; (b) a methylated sugar in the acceptor stem which is a region almost always free of modified residues; and (c) unusually weak base pairing in the dihydrouridine stem region consisting of only one standard A-U base pair in addition to GΨ, AC, and GU. This tRNA functions in a wheat germ cell free protein synthesizing system directed by either natural mRNAs or poly(G,U). The complete nucleotide sequence of wheat germ $tRNA_1^{Gly}$ is: pG-C-A-Cm-C-A-G-U-m^{1}G-G-U-C-Ψ-A-G-D-G-G-U-A-G-A-A-U-A-G-U-A-C-C-C-U-G-C-C-A-m^{5}C-G-G-U-A-C-A-G-A-m^{5}C-m^{5}C-m^{5}C-G-G-G-U-Ψ-C-G-m^{1}A-U-U-C-C-C-G-G-C-U-G-G-U-G-C-A-C-C-A_{OH}.

As our knowledge of transfer RNA broadens, it is becoming increasingly apparent that tRNAs are highly sensitive to changes in their primary structure. Although the primary structures of many tRNAs are now known, only very few of these are from higher organisms, and even fewer are from higher plants (Nishimura, 1974). While chromatographing crude wheat germ tRNA on BD-cellulose[1] columns, we became aware of the unusually early elution which was characteristic of the glycine acceptor tRNAs. Since unusual chromatographic properties often mirror unusual structural features, we examined these tRNAs further. We report elsewhere the isolation of each of the glycine tRNAs of wheat germ, their codon response, and their absence of ribothymidine (K. Marcu, D. Marcu, and B. Dudock, in preparation). We report here the isolation and complete nucleotide sequence of the major glycine tRNA of wheat germ and discuss several of its structural features.

Materials and Methods

Crude wheat germ tRNA containing about 10% ribosomal RNA and 5% 5S RNA was prepared as previously described (Dudock et al., 1969). Wheat germ tRNAs were fractionated by chromatography on BD-cellulose (Gillam et al., 1967) and on RPC-5 columns (Pearson et al., 1971) both run at neutral and acidic pH (Roe et al., 1973).

The preparation of crude aminoacyl-tRNA synthetases from

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[1] Abbreviations used: A_{260} unit, the quantity of material contained in 1 ml of solution which has an absorbance of 1 at 260 nm when measured in a 1-cm light-path cell; m^{1}A, 1-methyladenosine; m^{6}A, 6-methyladenosine; m^{5}C, 5-methylcytidine; Cm, *O*-methylcytidine; m^{1}G, 1-methylguanosine; m^{1}G>, cyclic 1-methylguanosine phosphate; D, dihydrouridine; T or rT, ribothymidine; BD-cellulose, benzoylated DEAE-cellulose; TLC, thin-layer chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SAM, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BAP, alkaline phosphatase; SVD, snake venom phosphodiesterase; NMR, nuclear magnetic resonance.