THE ISOLATION OF CHICKEN HISTONE F2c($\bar{v}$) MESSENGER RNA BY IMMUNOADSORPTION OF F2c-SYNTHESISING POLYSOMES

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SUMMARY: Immunoadsorbents and purified antibodies, prepared by affinity chromatography, have been used to specifically precipitate polysomes synthesising histone F2c from chicken reticulocytes. The 5-18S RNA fraction from these polysomes has been shown to programme the synthesis of F2c as the major detectable product in a wheat embryo cell-free translation system. The drug Trichodermin, an inhibitor of termination of eukaryotic protein synthesis increases the yield of immunoadsorbed messenger RNA.

INTRODUCTION

Although globin messenger RNA (mRNA) is readily prepared from avian reticulocytes by published procedures (1), we have never detected histone F2c mRNA activity in various size fractions of polysomal RNA, although synthesis of F2c occurs in these cells (2). Certainly, a far higher proportion of globin mRNA is expected and incubation of cells with [^{14}C]-leucine indicates that about 90% of the CCl_3COOH-precipitable radioactivity is accounted for as haemoglobin compared with about 4% in F2c (unpublished data) although these two proteins contain similar molar concentrations of this residue (3,4). Such a result is indicative of F2c being a relatively minor mRNA species. If, in addition, it does not contain a 3' poly A segment as reported for other histone mRNAs (5), then it is probably about the same size as globin mRNA, which does. These considerations led us to investigate an immunological approach to the isolation of F2c mRNA, particularly as this technique has been successfully applied in similar situations (6,7,8).

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This communication describes the use of immunoadsorbents and purified antibodies to specifically isolate the polysomes synthesising histone F2c. The RNA from these polysomes can be shown to programme the synthesis of F2c in an *in vitro* protein synthesising system. We also report on the use of the drug Trichodermin to increase the yield of mRNA produced in this fashion.

MATERIALS AND METHODS

Trichodermin was a kind gift of Dr. W.O. Godtfredsen of Leo Pharmaceutical Products Ltd., Ballerup, Denmark.

Preparation of Polysomes. Chicken reticulocytes obtained by published procedures (9) were collected into 0.15 M NaCl, 0.005 M KCl, 0.002 M $MgCl_2$, containing 0.1 mg/ml heparin and 1 µg/ml of Trichodermin. After washing and removal of the white cell layer, cells were lysed by addition of 2 volumes of 2 mM $MgCl_2$, 1 µg/ml Trichodermin for 2 minutes at 0°C before restoring tonicity with an equal volume of 0.01 M Tris-HCl pH 7.6, 0.2 M KCl, 2 mM $MgCl_2$. The lysate was centrifuged at 12,000 g for 10 minutes, the supernatant removed, and centrifuged again. The final supernatant was layered over $^1/_5$ volume of 50% sucrose in 0.01 M Tris-HCl pH 7.6, 0.1 M KCl, 2 mM $MgCl_2$, 1 µg/ml Trichodermin, and centrifuged at 50,000 r.p.m. for 60 minutes, at 2°C, in a Beckman Ti50 rotor. The polysome pellet was carefully rinsed with 0.025 M Tris-HCl pH 7.6, 0.025 M NaCl, 0.005 M $MgCl_2$ containing 1 µg/ml Trichodermin and 0.1 mg/ml heparin, and suspended in the same buffer.

Histone Purification and Matrix Preparation. Chicken histone isolation was based on the procedures of Dingman and Sporn (10), as modified by Appels, Wells and Williams (9). Histones F1 and F2c were prepared by precipitation of all other histones with 5% perchloric acid. F2c was then purified to electrophoretic homogeneity by chromatography on a Whatman CM52 carboxymethyl cellulose column using a gradient of 0.2 to 0.8 M NaCl in 0.1 M Sodium Formate buffer pH 4.7.

Glutaraldehyde cross-linked histone matrix was prepared according to Palacios *et al* (6), with the modification that the protein concentration was increased four-fold.

Antibody Purification. Antibodies to F2c were induced in rabbits by five injections of 3 mg of purified F2c in complete Freund's adjuvant at 2-week intervals. The antiserum was rendered specific by passage through affinity columns prepared according to March, Parikh and Cuatrecasas (11). The antiserum was first passed through a column containing chicken haemoglobin linked to sepharose 4B to remove any anti-globin activity.

The titre was then increased, and specificity ensured by passage through a column of F2c-Sepharose by the technique of Shapiro *et al* (7). Ribonuclease activity was removed by passage through carboxymethyl cellulose then DEAE-cellulose according to Palacios *et al* (6).

Immunoprecipitation. Immunoprecipitation was carried out as described by Palacios *et al* (6) with the following exceptions. The polysome suspension contained 1 µg/ml Trichodermin and only 25 µg of purified antibody to F2c was used per 10 A_{260} units of polysomes. Precipitation was induced by the addition of 20 mg of total histone matrix per 10 A_{260} units. The final ethanol precipitated, F2c-synthesising polysomes were taken up in 0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 0.05 M EDTA, 0.1% SDS, and loaded onto 10-40% sucrose gradients made up in the same buffer without SDS. The gradients were centrifuged at 40,000 r.p.m. for 16 hours, at 2°C, in a Beckman SW41 rotor, and fractionated in an ISCO density gradient fractionator. The fraction shown in figure 1 was collected, ethanol precipitated, and used in translation experiments.

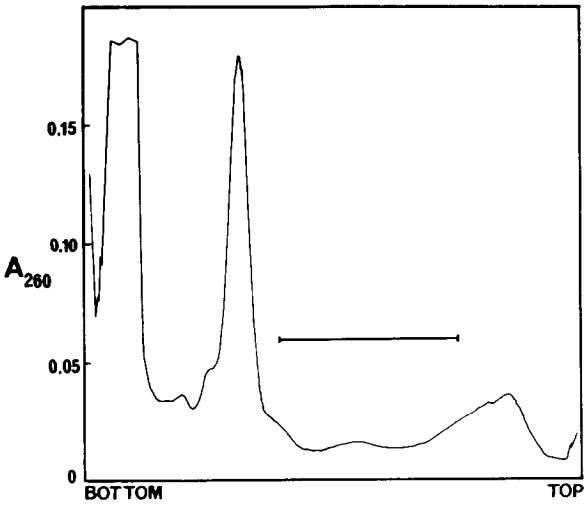

Figure 1. Sucrose gradient profile of RNA eluted from immunoadsorbent (Materials and Methods). The bar denotes the 5-18S RNA which was collected and used as mRNA in all translation experiments.

Cell-Free Translation. The conditions of the cell-free translations were similar to those of Shih and Kaesberg (12). Each mixture contained, in a final volume of 50 µl, 25 mM Tris-Acetate pH 8.0, 80 mM KCl, 3.8 mM Magnesium acetate, 2.3 mM Dithiothreitol, 1 mM ATP, 0.25 mM GTP, 8 mM Creatine Phosphate, 4 µg of creatine phosphate kinase, 1 µCi of [^{3}H]Leucine (13 Ci/mmole), mRNA as required, and 30 µl of wheat embryo extract. Incubation

was at 30°C for 1 hour. After this time, the tRNA was deacylated by incubation with 0.1 M NaOH at 37°C for 12 minutes. The reaction mixture was then precipitated and washed with trichloroacetic acid according to Boime, Aviv and Leder (13). Precipitated samples were either collected onto GF/A filter papers for determination of radioactivity, or taken up directly in loading buffer for electrophoresis on 0.6 x 8 cm polyacrylamide gels with [^{14}C] *in vivo* labelled standards. The gels used were either the SDS-Urea gels of Swank and Munkres (14), or the pH 2.8 gels of Panyim and Chalkley (15). After electrophoresis, the gels were sliced into 1 mm slices and radioactivity eluted with 0.2 ml of 0.5% SDS for 16 hours. 2 ml of toluene-Triton X-100 scintillant was then added and the radioactivity determined in a Packard Tri-Carb Liquid Scintillation Counter.

RESULTS AND DISCUSSION

F2c is the only histone synthesised in the non-dividing chicken reticulocyte (2). Whatever the biological significance of this may be, it is particularly advantageous, practically, since it allows us to use total histone for the preparation of the protein matrix. Since total histone is easily prepared in high yield, it overcomes one of the major drawbacks of the immunoadsorption technique, viz the need to use large amounts of purified protein to prepare the matrix. The amount of matrix to add, 20 mg per 10 A_{260} units of polysomes, is determined on an empirical basis. The addition of more matrix did not increase the yield of immunoadsorbed RNA. We have further shown that the addition of more antibody than 25 µg per 10 A_{260} units of polysomes (Materials and Methods) will increase the amount of matrix required, however, this has little effect on the yield of RNA (data not shown). Indeed, any increase in the size of the precipitate is to be avoided since this decreases the specificity of the method by increasing the amount of non-specifically bound polysomes (6).

As shown in figure 2, some 70% of the [^{3}H]Leucine incorporated in a cell-free system programmed with immunoadsorbed RNA, co-electrophoreses with [^{14}C]F2c standard on two different

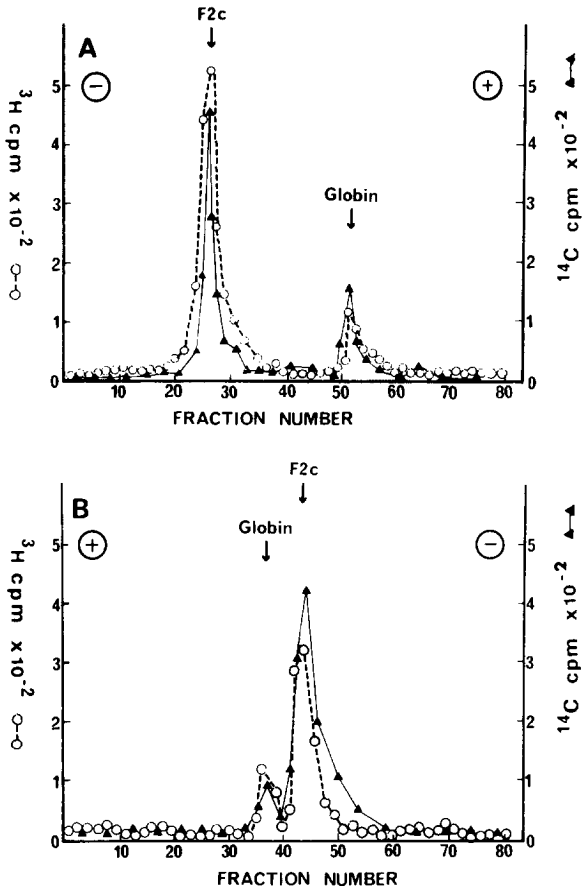

Figure 2. Analysis of the products of *in vitro* translation of putative F2c mRNA, on polyacrylamide gels. TCA-precipitated products of the *in vitro* translations were suspended in loading buffer and mixed with [^{14}C]labelled standards. This consisted of 5000 cpm of [^{14}C] F2c and 1400 cpm of [^{14}C]globin, both electrophoretically pure. The products are run on A, SDS-Urea gels, and B, pH 2.8 gels, then sliced and examined for radioactivity (Materials and Methods). The [^{14}C] counts accounted for 10% spillover into the [^{3}H] channel and this was subtracted. Electrophoresis is from left to right in both cases.

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gel systems. The rest of the radioactivity largely co-electrophoreses with globin in both systems. As stated before, the amounts of haemoglobin and F2c synthesised relative to total protein synthesis is about 90% and 4% respectively, and although only an approximation, we use these figures as an estimate of

the percentage of mRNA in both cases. On this basis, we have increased the percentage of F2c mRNA relative to globin mRNA some 17-fold. Using these approximations, it is possible to estimate the specificity of the method by determination of the extent of globin mRNA (and hence globin-synthesising polysomes) compared with F2c mRNA activity derived from immunoprecipitated polysomes. This is calculated using the data in table I which shows the amount of material obtained at various stages in the preparation of mRNA, with and without Trichodermin. Starting with 640 A_{260} units of polysomes, 6.8 A_{260} units are eluted from the matrix containing 1 μg/ml Trichodermin. If 25% of these are synthesising globin (figure 2), then we have 1.70 (i.e., 25% of 6.8) A_{260} units non-specifically bound out of an original 576 (90% of 640) A_{260} units synthesising globin *in vivo*. Thus the contamination with non-specifically bound polysomes

$$= \frac{\text{Amount of globin-synthesising polysomes bound}}{\text{Total globin-synthesising polysomes originally present}}$$

$$= \frac{1.7}{576} \times 100 \quad \%$$

$$= 0.3\%$$

This figure assumes that the polysomes synthesising F2c and globin are of the same average size and therefore may be a minimum estimate, but it compares favourably with the 2% contamination reported by Palacios *et al* (6) using the same technique. Those authors found that the percentage contamination depended very markedly on the size of the immune precipitate. In this case, we used very small amounts of antibody since it is highly purified and is directed against only some 4% of the polysomes, i.e., those making F2c. The amount of matrix necessary to precipitate this amount of antibody is correspondingly small, and therefore the final precipitate is also.

TABLE I
EFFECTS OF VARIOUS LEVELS OF TRICHODERMIN ON THE YIELD OF IMMUNOADSORBED POLYSOMES AND MESSENGER RNA

Level of Trichodermin (μg/ml)	A_{260} units of polysomes eluted from matrix	Total mRNA activity in 5-18S RNA (total cpm)
0	2.4	9700
0.2	3.4	12400
1.0	6.8	19100

The original amount of polysomes used in each case was 640 A_{260} units. All RNA samples were translated at non-saturating concentrations.

The effect of Trichodermin on the isolation is clearly seen in table I. The yield of A_{260} units eluted from the matrix and of mRNA extracted (as judged by capacity to stimulate translation) are both increased with the addition of Trichodermin. Chicken polysomes will run down rapidly during isolation unless a protein synthesis inhibitor such as Trichodermin or cycloheximide is present (16, J.R.E. Wells, unpublished). Trichodermin is a potent inhibitor of termination of protein synthesis (17) in eukaryotic cells. Hence in its presence, polysomes are larger with correspondingly more and longer nascent peptides per mRNA molecule. Presumably, this gives a greater degree of binding of antibody to the polysomes and thereby increases the yield and posssibly the specificity of the immunoadsorption reaction.

We have therefore made use of immunological methods to effect a substantial purification of the mRNA for chicken histone F2c. The drug Trichodermin has been demonstrated to be useful in increasing the yield of mRNA produced by these methods.

While histone mRNA has been isolated by size fraction of polysomal RNA from HeLa cells synchronised in S-phase (18) or from cleavage embryos (19) in which it is a relatively major polysomal component, to our knowledge this is the first reported isolation of F2c mRNA activity. Work is in progress to further purify and characterise this tissue-specific mRNA .

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