THE ENRICHMENT OF CHICKEN EMBRYO tRNA GENES IN NUCLEOSOMAL DNA BY REVERSED PHASE CHROMATOGRAPHY

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1. Introduction

The RPC-5 reversed phase chromatography system is well known in the high resolution fractionation of tRNA into individual tRNA species [1–3]. The system combines phase distribution effects on a hydrophobic polychlorotrifluoroethylene matrix with ion exchange chromatography accomplished by the tricaprylylmethylammonium groups coated onto the matrix. It follows that besides common ionic binding forces the extent of secondary structure affecting the accessibility of purine- and pyrimidine-base charges modulates the influence of each of the two chromatographic effects on a particular class of nucleic acids. In the RPC-5 chromatography of restriction fragments of double stranded DNA a number of fragments elute at positions not expected in view of their molecular weight, i.e., their ionic net charge, signalling the influence of the phase distribution effect [4,5]. RNAs with little or no secondary structure elute preferentially in order of their size under the main influence of ion exchange forces from RPC-5 columns [6].

Here we report on two methods for the enrichment of tRNA genes in nucleosomal DNA employing extensions of both chromatographic effects of RPC-5:

1. DNA of 570 basepairs isolated from nucleosome trimers [7] was fractionated by RPC-5 using concentration gradients of the G·C-specific dye phenyl neutral red [8] and of sodium acetate, simultaneously. Assaying the effluent DNA fractions for hybridization with ^{125}I-labelled chicken embryo total tRNA revealed distinct peaks of radioactivity. Hybridization with single species tRNA allowed the assignment of some of these peaks to a specific tRNA. Genes for a specific tRNA are >100-fold enriched in peak fractions.
2. We found that RPC-5 chromatography at 70°C efficiently separates tRNA : DNA hybrids from non-hybridized double stranded DNA and tRNA, respectively. Hybridization of total chicken embryo tRNA with nucleosomal DNA 60–90-fold enriched for tRNA genes by method 1 followed by isolation of the tRNA : DNA hybrids yields approximately pure preparations of single stranded tRNA gene containing DNA.

2. Experimental

Phenyl neutral red (PNR) was a gift from Bayer AG. DNA from nucleosome di-, tri- and tetramers was isolated from day 13 chicken embryos [7]. Total tRNA and single species tRNAs were isolated and purified from the same animal source [3]. tRNA was ^{125}I-labelled to spec. act. 5×10^6 cpm/μg [9] and nucleosomal DNA was 5′-end [^{32}P]-labelled to 6×10^3 cpm/μg [10].

For reversed phase chromatography in the presence of PNR a 140 × 0.6 cm RPC-5 column was equilibrated at 35°C with 1.4 M Na-acetate, 0.5 mM EDTA, 10 mM Tris-acetate (pH 7.3) containing 1 mg/l PNR. The flow was 0.8 ml/min at 2.5–3.0 MPa. DNA was dialysed against equilibration buffer without PNR and loaded onto the column in 1–2 ml (A_{260} = 200–300). A decreasing concentration

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gradient of PNR together with an increasing salt concentration gradient were formed by mixing equilibration buffer with 1.75 M Na-acetate, 0.5 mM EDTA, 10 mM Tris-acetate (pH 7.3) under control of an Ultrograd Gradient Mixer (LKB Producter). Gradient volume was 500 ml, fractions of 2.5 ml were collected.

High temperature RPC-5 chromatography for the isolation of tRNA : DNA hybrids was performed using a 0.25 × 35 cm column equilibrated with 1.4 M Na-acetate, 0.5 mM EDTA, 10 mM Tris-acetate (pH 7.3) at 70°C. For the preparation of chromatographic samples hybridization mixtures contained (a) in 1 ml: 1.5 μg/ml ^{125}I-labelled total tRNA, 1 mg/ml nucleosomal DNA, 2 × SSC, 0.2% SDS, 0.5 mM EDTA or (b) in 0.1 ml: 15 μg/ml ^{125}I-labelled total tRNA, 1 mg/ml DNA 60–90-fold enriched for tRNA genes, and other ingredients as before. Incubation was at 65°C for 16 h, the R_0t values reached were (a) 0.247 and (b) 2.47 M. s. Samples were precipitated with ethanol, dissolved in 100 μl equilibration buffer and loaded onto the column. A non-linear 1.4–1.75 M Na-acetate gradient in equilibration buffer was applied. Gradient volume was 40 ml. The flow was 0.08 ml/min at 1.5 MPa. Fractions of 0.2 ml were collected.

Fractionated DNA was re-hybridized in 60 μg assays containing 1.5 μg/ml ^{125}I-labelled total tRNA or 0.7 μg/ml ^{125}I-labelled single species tRNA, 1–1000 μg/ml DNA (depending on the tRNA gene enrichment), 2 × SSC, 0.2% SDS, 0.5 mM EDTA. Incubation was at 65°C for 16 h. Samples were diluted 10-fold with 0.2 M NaCl, 4 mM EDTA, 20 mM Tris–HCl (pH 7.2) digested with RNase A and T_1 (10 μg/ml and 250 U/ml, respectively) and processed for liquid scintillation counting.

3. Results

Figure 1A shows the RPC-5 chromatography of DNA (~570 base pairs) from nucleosome trimers under conditions where an increasing gradient of sodium acetate concentration alone was used to elute the DNA. A tailing DNA peak is eluted which has few or no sequences complementary to ^{125}I-labelled total tRNA in its front fractions, whereas strong hybridization is found broadly distributed in

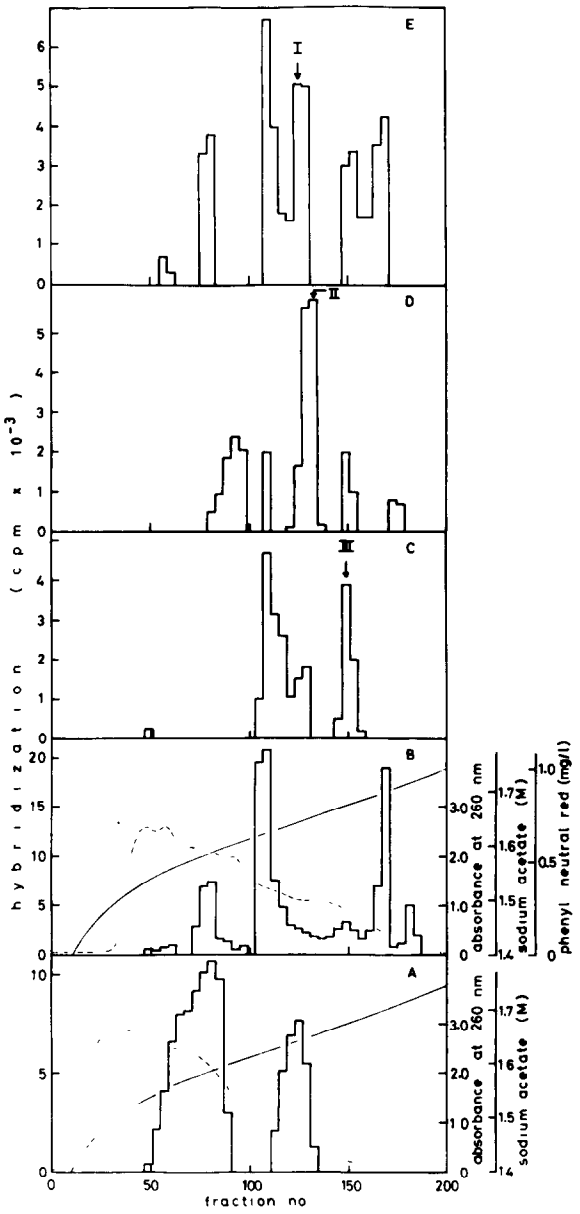

Fig.1. RPC-5 chromatography of DNA from nucleosome trimers and hybridization with tRNA. (A, B) Hybridization assayed with ^{125}I-labelled total tRNA. Background subtracted: 5300 cpm. (C–E) Fractions of (B); hybridization assayed with ^{125}I-labelled $tRNA_2^{Lys}$ (C), $tRNA_4^{Lys}$ (D) and $tRNA^{Phe}$ (E). Background subtracted: 2600 cpm. (- - - -) Absorbance; (———) sodium acetate concentration; (· · · ·) phenyl neutral red concentration; bars, cpm hybridized. Column fractions each were precipitated with ethanol, dissolved in H_2O to A_{260} 30, and aliquots hybridized with the respective ^{125}I-labelled tRNA; every 4th fraction was assayed. Numbered arrows, see section 3.

the tail fractions. When besides the sodium acetate concentration gradient a decreasing gradient of the G·C-specific dye PNR was employed, simultaneously, the profile of ultraviolet-absorbing material is similar although the DNA is eluted considerably later (fig.1B). However, hybridization in the effluent fractions reveals clearly resolved peaks of radioactivity in the tail of the DNA peak. Although not shown here it should be mentioned, that DNA from nucleosome dimers and tetramers (~380 and 760 basepairs, respectively) elutes at the same concentration of salt and phenazinium dye as the 570 basepairs DNA and that hybridization yields essentially the same results.

Assaying for sequences complementary to $tRNA_2^{Lys}$, $tRNA_4^{Lys}$ and $tRNA^{Phe}$ (fig.1C–E) shows different radioactivity profiles for all the 3 labelled tRNA species. One individual peak of hybridization (marked by numbered arrows, fig.1C–E) can be recognized for each of the 3 single species tRNAs which is not found or only hinted in the hybridization with total tRNA (fig.1B). Since in all experiments excess labelled tRNA was present a comparison of the depicted peak heights are direct measures of tRNA gene enrichment in a particular fraction. Estimating that in chicken embryos roughly 1000 genes code for ~100 different tRNA species, tRNA genes are enriched 150-, 300- and 100-fold in peak fractions I, II, and III (fig.1C,D,E), respectively, compared to the hybridization measured with total tRNA (fig.1B).

Figure 2 shows the RPC-5 chromatography of a mixture of ^{32}P-labelled nucleosomal DNA and ^{125}I-labelled tRNA at 35°C and 70°C, respectively. At 35°C both components cannot be separated from each other (fig.2A) whereas at 70°C (fig.2B) the DNA elutes as a sharp peak at the front of the gradient and the tightly bound tRNA leaks from the column near the end. A 2.0 M Na-acetate wash is required to elute the tRNA quantitatively.

Figure 3 gives an explanation for this change. Midpoints (T_m) of the thermal denaturation curves of tRNA and nucleosomal DNA differ from more than 25°C. Hence, most of the tRNA will have lost its base paired structure, when nucleosomal DNA is still double stranded.

Chromatography of the components of a hybridization mixture of ^{125}I-labelled tRNA and DNA from

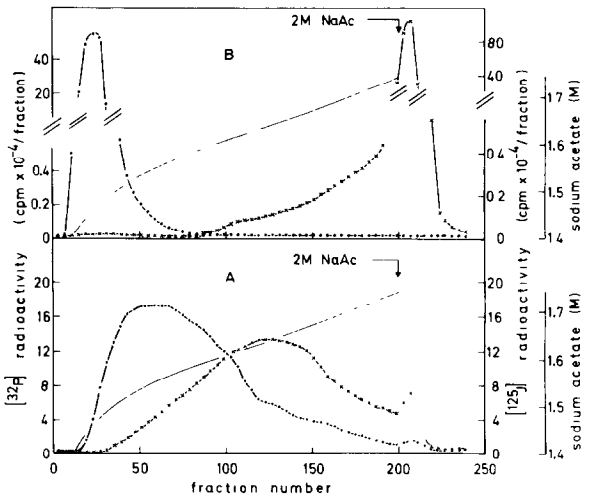

Fig.2. RPC-5 chromatography of mixtures of ^{125}I-labelled tRNA and 5′-end ^{32}P-labelled nucleosomal DNA. (A) 8×10^6 cpm of ^{125}I-labelled tRNA together with 6×10^6 cpm [^{32}P]DNA from nucleosome trimers; chromatography at 35°C. (B) Same sample as in (A), chromatography at 70°C. (•–•) ^{32}P radioactivity; (x-x) ^{125}I radioactivity; (———) sodium acetate.

nucleosome trimers (~570 basepairs) at 70°C is shown in fig.4A. Again DNA elutes into front fractions and the labelled tRNA near the end, but additionally a broad distribution of ^{125}I-label is

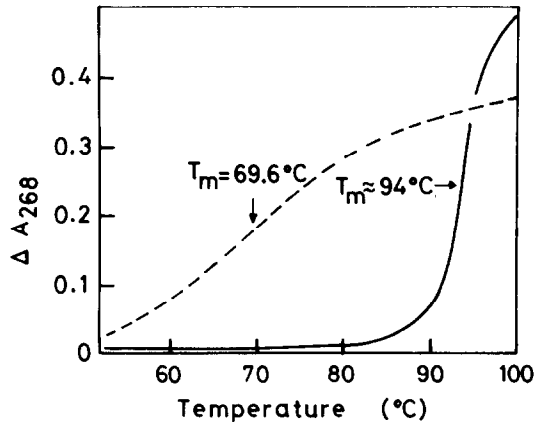

Fig.3. Thermal denaturation curves of tRNA and nucleosomal DNA. (– – – –) Total tRNA (A_{260} 1.16); (———) DNA from nucleosome trimers (A_{260} 1.04) in 1.4 M Na-acetate, 0.5 mM EDTA, 10 mM Tris-acetate (pH 7.3).

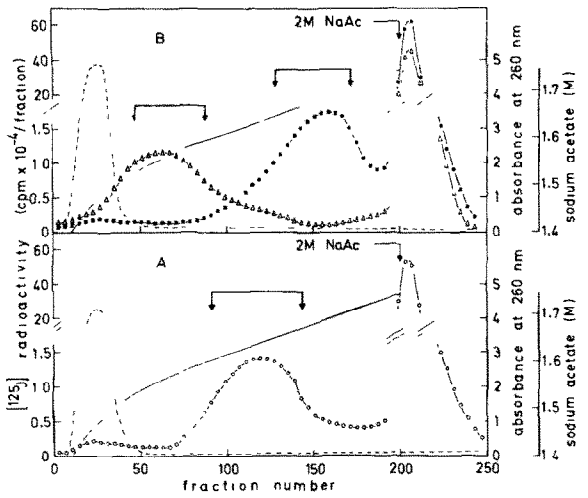

Fig.4. RPC-5 chromatography of hybridization mixtures of ^{125}I-labelled tRNA and DNA from nucleosome di-, tri- and tetramers. (A) Hybridization mixture containing DNA from nucleosome trimers. (———) A_{260}; (○—○) ^{125}I radioactivity. (B) Hybridization mixture containing DNA from nucleosome dimers: (———) A_{260}; (■—■) ^{125}I radioactivity. Hybridization mixture containing DNA from nucleosome tetramers: (△—△) ^{125}I radioactivity; (———) sodium acetate concentration.

found in fractions no. 75–150. A similar distribution of ^{125}I-label elutes into fractions no. 110–185 upon high temperature chromatography of hybridization mixtures containing DNA from nucleosome dimers (380 basepairs) or into fractions no. 25–105 when DNA from nucleosome tetramers (760 basepairs) is employed (fig.4B).

Supposing that this distribution of radioactive label represents tRNA:DNA hybrids, we pooled the respective fractions (marked with arrows in fig.4), hydrolysed the tRNA, and rehybridized the remaining DNA (1–2% of starting DNA) with ^{125}I-labelled tRNA (table 1). The hybridization data clearly indicate that DNA complementary to tRNA is enriched in these fractions. The enrichment depends on the length of the nucleosomal DNA. Possibly the background of double stranded DNA increases toward the front of the salt concentration gradient (fig.2B) and interferes with the purity of the hybrids. Starting with nucleosomal DNA already enriched for tRNA genes by RPC-5 chromatography in the presence of PNR tRNA:DNA hybrid isolation by high temperature RPC-5 chromatography led to approximately pure preparations of tRNA gene containing single stranded DNA.

4. Discussion

Employing simultaneous concentration gradients of salt and phenyl neutral red tRNA genes in nucleosomal DNA of homogeneous length can be effectively fractionated by RPC-5 chromatography. The dye

Table 1
tRNA gene enrichment in nucleosomal DNA

DNA starting material		DNA obtained		
Hybridization (cpm/mg DNA) × 10^{-5}	Enrichment factor	Hybridization (cpm/mg DNA) × 10^{-5}	Enrichment factor	Total enrichment factor
N_2 4.23	1	406	96	96
N_3 4.68	1	388	83	83
N_4 4.62	1	217	47	47
N_2 enr. 271	64	14 630	54	3458
N_3 enr. 430	92	21 070	49	4502
N_4 enr. 393	85	14 148	36	3062

N_2, N_3, N_4 refers to DNA from nucleosome di-, tri- and tetramers, respectively; N_2 enr., N_3 enr., N_4 enr. refers to the respective nucleosomal DNA enriched for tRNA genes by RPC-5 chromatography in the presence of PNR

concentration in our column equilibration buffer is more than an order of magnitude lower than the concentrations used in [11] for hydroxyapatite chromatography. It has, however, still to be reduced continuously during chromatography to allow for desorption at practicable salt concentrations but not to abolish the high resolution of tRNA genes. When restriction endonuclease generated fragments (*Alu* I and *Hha* I) are fractionated in the presence of PNR (not shown here) some fragments change their elution position compared to chromatography without the dye concentration gradient. This could be useful for the purification of a given fragment.

The rationale of the tRNA:DNA hybrid isolation procedure was to perform RPC-5 chromatography at such a temperature which: (a) denatures the tRNA tertiary and secondary structure to enhance tRNA·resin binding by ion exchange forces; (b) disrupts the hydrophobic interactions which adsorb the double stranded DNA; (c) does not denature the double stranded DNA since the single stranded DNA behaves similarly like tRNA upon RPC-5 chromatography [12]. 70°C seems to be a good compromise (fig.3), since tRNA is bound strongly enough and practically no unspecific single stranded DNA contaminates the tRNA:DNA hybrids.

However, we expected hybrids of tRNA and long DNA molecules, e.g., the 760 basepairs DNA, to elute later from the column, in view of the strong binding of the long single stranded part of DNA in such hybrids. Since, as demonstrated, the contrary happens, we suppose that besides tRNA:DNA hybridization also DNA:DNA association occurs with these hybrids. Preliminary studies on the purification of hybrids between tRNA and DNA restriction fragments longer than 1 kilobase revealed that hybridization should be performed at high formamide concentration [13] to minimize DNA:DNA reassociation. Under these conditions the method should also be useful for the isolation of mRNA:DNA hybrids.

Assuming the average length of a tRNA gene to be 100 basepairs [14], ~0.02% of the chicken embryo nucleosomal DNA is complementary to tRNA. It follows that 60–90% of the DNA finally obtained contains tRNA complementary sequences.

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