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ISOLATION AND CHARACTERIZATION OF PROLACTIN-COPY DNA

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Summary: The novel properties of rat pituitary tumor cells (GH-cells) in culture have been utilized to isolate prolactin-copy DNA ($cDNA_{PRL}$). The $cDNA_{PRL}$ has been characterized by i) polyacrylamide gel electrophoresis ii) by sedimentation on alkaline sucrose gradients and by iii) the kinetic study of the reassociation of "$cDNA_{PRL}$" with polysomal RNA and with polysomal poly(A) RNA isolated from cells which contain $mRNA_{PRL}$ in abundance and from cells which do not contain any translatable $mRNA_{PRL}$ sequences. The $cDNA_{PRL}$ fraction reassociated with polysomal RNA and polysomal poly(A) RNA isolated from PRL producing cells (PRL^+) with pseudo first order kinetics, whereas no significant reassociation was observed when the $cDNA_{PRL}$ was hybridized with the same two RNA fractions isolated from PRL-nonproducing cells (PRL^-). The [^3H] $cDNA_{PRL}$ moved as a sharp band of radioactivity when analyzed by polyacrylamide gel electrophoresis and sedimented in alkaline sucrose gradients in the size range of 6.7s. A mRNA fraction which is highly enriched for PRL-specific mRNA ($mRNA_{PRL}$) has been isolated from PRL^+ cells. The $cDNA_{PRL}$ hybridized with $mRNA_{PRL}$ with an $eRot_{\frac{1}{2}}$ value of 0.007-0.008. From these results it is calculated that more than 75% of the $mRNA_{PRL}$ enriched fraction contains $mRNA_{PRL}$ sequences.

Different clonal strains of rat pituitary tumor cells in culture (GH-cells) synthesize and secrete into the medium different amounts of prolactin (PRL) and growth hormone (GH). Both PRL and GH production in these cells may be further modulated by physiological agents such as thyrotropin releasing hormone (TRH), hydrocortisone (HC), and estradiol (E_2) (1). One of the clonal strains, GH_12C_1, does not produce detectable amounts of PRL and these cells do not contain any translatable $mRNA_{PRL}$ (2). These cells are designated as PRL^- cells. A second clonal strain, GH_4C_1, produces PRL, and is thus designated as PRL^+. These PRL^+ cells can be induced to produce larger quantities of PRL in the presence of TRH. Thus the major difference in the population of translatable mRNA between these two isogenic strains is the presence of $mRNA_{PRL}$ in abundance in PRL^+ cells and its absence in PRL^- cells. This novel property of these two GH-cell strains has been exploited to isolate and characterize $cDNA_{PRL}$. The single stranded DNA

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copies made from polysomal poly(A) RNA of PRL^+ cells by the use of viral reverse transcriptase will contain DNA copies of all sequences common to PRL^+ and PRL^- strains as well as $cDNA_{PRL}$. Molecular hybridization of the single stranded DNA fractions prepared from the PRL^+ cells with polysomal poly(A) RNA isolated from the PRL^- strain under optimum hybridization conditions should therefore convert all the cDNA into double stranded hybrids with mRNA, except the $cDNA_{PRL}$. $cDNA_{PRL}$ has been isolated following such a scheme (scheme I, Fig. 1) and the characteristics of this $cDNA_{PRL}$ preparation are presented in this report. This scheme provides an efficient method for the isolation of $cDNA_{PRL}$ without prior isolation of $mRNA_{PRL}$.

Methods: The growth conditions for the two GH-cell strains have been described previously (2). Cells were harvested, washed with buffer and polysomes were isolated as described earlier (2).

The poly(A) RNA from polysomes was isolated by phenol extraction and subsequent oligo(dT) cellulose chromatography(3). In some experiments RNA was made radioactive by incubation of cells in suspension culture with 1mCi [^{32}P] orthophosphoric acid/10^9 cells for 4 hr at 37°.

Synthesis of cDNA and oligo (dT) cellulose bound cDNA from polysomal poly(A) RNA: cDNA from polysomal poly(A) RNA extracted from PRL cells (scheme I, Fig. 1) and oligo (dT) cellulose bound cDNA from the RNA preparation extracted from PRL^- strain (scheme II, Fig. 1)were prepared as follows. Reaction mixtures for the synthesis of cDNA contain 50 mM tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 1.0 mM each of dATP, dGTP, dTTP and dCTP (in experiments where radioactive cDNA was made, dGTP concentration was 0.1 mM and the reaction mixture also contained 100 µCi/ml [^{3}H]dGTP), 10 µg/ml poly(A) RNA, 100µg/ml actinomycin D, 100-200 units/ml of AMV reverse transcriptase (obtained from Dr. J.W. Beard of Life Sciences, Inc.) and 10-50 µg/ml of oligo (dT) or 30 mg/ml oligo (dT) cellulose.

In the case of cDNA prepared from poly(A) RNA of PRL^+ cells (scheme I, Fig. 1), after the incubation the reaction was made 0.5% with SDS and the EDTA concentration was adjusted to 10 mM. The sample was then passed through a Sephadex G-50 column equilibriated with buffer containing 0.1 M NaCl, 2 mM EDTA, 0.01 M tris-HCl, pH 7.0. The excluded fraction was collected and made 0.1 M with NaOH and heated at 60° for 30 min. The pH of the mixture was readjusted to 7.0 and the sample was dialyzed extensively against dH_2O and lyophilized. The amount of [^{3}H] cDNA synthesized generally corresponds to 10-15% of the poly(A) RNA used in the reaction mixture as template.

In the case of synthesis of matrix bound cDNA (scheme II, Fig. 1), after shaking the reaction mixture vigorously for 90 min in a 37° water bath, the reaction was terminated and RNA removed by treatment with 0.1 M NaOH and the cellulose was washed with water. In a typical experiment synthesis of cDNA covalently bound to cellulose was dependent on the amount of poly(A) RNA used as template. The amount of cellulose bound cDNA synthesized generally correspond to 5-10% of the poly(A) RNA used in the reaction mixture as template.

Hybridization of poly(A) RNA to cDNA (as prepared by following scheme I, Fig.1) and separation of single stranded cDNA from cDNA/mRNA hybrids: The reaction mixtures containing 0.25 M phosphate buffer, pH 6.9, 5 mM EDTA, 50-100 pg [^{3}H]cDNA and 10-100 µg/ml polysomal poly(A) RNA were denatured at 100° for 2 min and incubated for 40 hr in a sealed plastic conical tube submerged in a water bath at 60°. After the incubation, the reaction mixture was then adjusted to 0.125 M

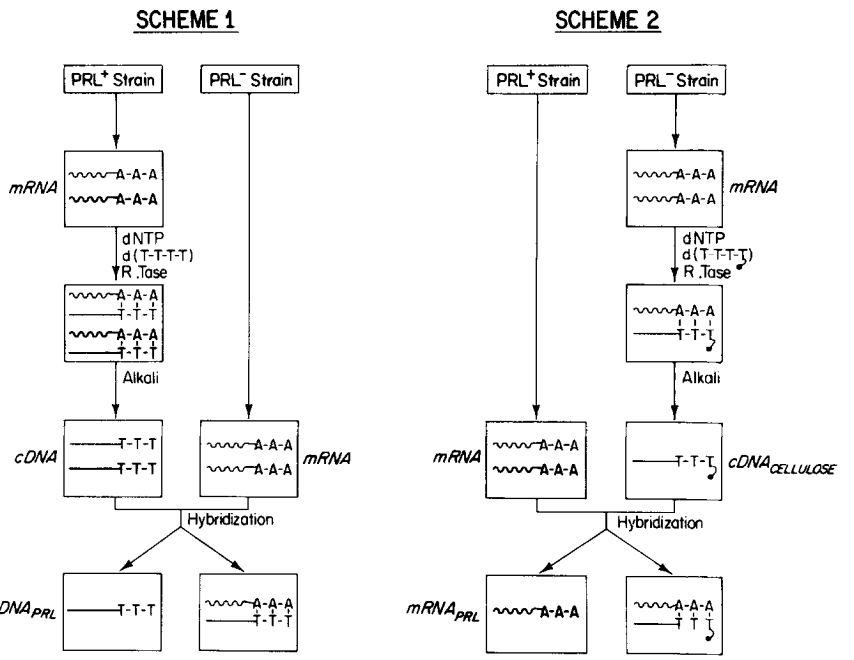

Fig. 1. Schemes for the preparation of $cDNA_{PRL}$ and $mRNA_{PRL}$. PRL^+= Prolactin producing cells; PRL^-= Prolactin nonproducing cells. Isolation of polysomal poly(A) RNA, preparation of cDNA and hybridization conditions are described in Methods. The separation of single stranded cDNA and cDNA/mRNA hybrids on hydroxylapatite column is described in Methods.

with respect to phosphate and applied to a hydroxylapatite column at 60°, previously equilibriated with 0.125 M phosphate buffer, pH 6.9. The single stranded [^{3}H] cDNA fraction does not bind to the column. The hybridized RNA/DNA duplex can be eluted with 0.33 M phosphate buffer. The single stranded [^{3}H]cDNA fraction in the flow through was then dialyzed against dH_2O and lyophilized.

Hybridization of poly(A) RNA to cDNA cellulose (scheme II, Fig. 1): Optimum conditions for matrix bound cDNA/RNA hybridization are defined as those conditions which permit maximum (90-100%) binding of poly(A) RNA species to cDNA-cellulose prepared in the presence of reverse transcriptase using the same RNA species as template. The conditions are as follows: after calculation of the cDNA synthesized, poly(A) RNA was added to the cDNA cellulose so that cDNA was present in the reaction mixture in excess. The hybridization was performed in buffer containing 50% formamide, 10 mM tris-HCl, pH 7.5, 0.6 M NaCl, 10mM EDTA. The poly(A) RNA was incubated at 70° for 5 min with cDNA cellulose, followed by an additional 30-60 min incubation at 40°. Unbound material is removed by washing with hybridization buffer at room temperature. The cDNA cellulose was then washed with 10 mM tris, pH 7.5 at 4° to remove non-specifically bound material. Hybridized RNA species were eluted with 10 mM tris, pH 7.5 at 70°. Throughout the procedure, oligo (dT) cellulose alone was used as a control for the determination of the specific hybridization and elution of poly(A) RNA. In a typical experiment 90% of the poly(A) was recovered from the cDNA cellulose column. Very little (10%) binding to cellulose was observed under the optimum hybridization conditions.

Results and Discussion: The unhybridized single stranded cDNA fraction obtained following the procedure described in scheme I, and the unhybridized mRNA fraction

obtained by following the procedure described in the scheme II of Figure 1 have been analyzed by polyacrylamide gel electrophoresis. The cDNA preparation has also been characterized by sedimentation in alkaline sucrose gradients and by rehybridization studies with RNA fractions obtained from PRL^+ and PRL^- strains. Results presented in Fig. 2A show the electrophoretic mobility of [^{3}H] cDNA species prepared from total polysomal poly(A) RNA of TRH treated cells before (•-•) and after (o-o) hybridization with total polysomal poly(A) RNA isolated from PRL^- cells as described in scheme I (Fig. 1). These results show that the unhybridized single stranded [^{3}H]cDNA ($cDNA_{PRL}$) moves as a sharp band of radioactivity during polyacrylamide gel electrophoresis. This batch of cDNA had a specific activity of $7x10^6$ cpm/μg DNA. The results presented in Fig. 2B show the sedimentation profile of [^{3}H]$cDNA_{PRL}$ as observed after alkaline sucrose gradient centrifugation. The size of this cDNA preparation is approximately 6.7s as determined by comparing its sedimentation to that of DNA markers of known sizes. The total population of [^{3}H]cDNA prepared from the total polysomal poly(A) RNA of GH_4C_1 cells was found to be heterogenous in size and sedimented between 4.9s and 16s (results not shown).

Results presented in Fig. 2C show the electrophoretic mobility of the mRNA population of the PRL^+ strain before (•-•) and after (o-o) hybridization with matrix bound cDNA prepared from total poly(A) RNA of the PRL^- strain. The unhybridized mRNA fraction migrates as a relatively sharp band compared to the total heterogenous mRNA fraction suggesting that this unhybridized fraction is enriched for specific mRNA, i.e. $mRNA_{PRL}$.

Results presented in Fig. 3 show the reassociation kinetics of [^{3}H] $cDNA_{PRL}$ with total polysomal RNA (Fig. 3A), with polysomal poly(A) RNA (Fig. 3B) and with $mRNA_{PRL}$ (Fig. 3C). The $cDNA_{PRL}$ fraction hybridized with total polysomal RNA (Fig. 3A, •-•) and polysomal poly(A) RNA (Fig. 3B, o-o) from PRL^+ cells, both of which contain abundant $mRNA_{PRL}$ sequences, with pseudo first order kinetics and an $eRot_{½}$ of about 15 and 0.7 respectively. The $cDNA_{PRL}$ did not reassociate with either total polysomal RNA (Fig. 3A, o-o), or with polysomal poly(A)

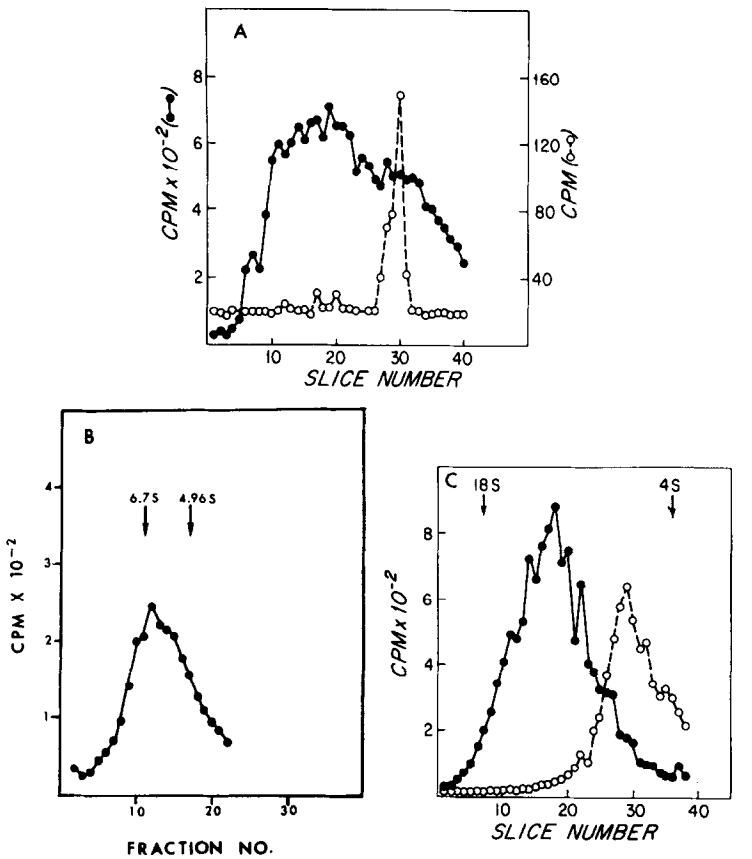

Fig. 2A. Polyacrylamide gel electrophoresis of cDNA fractions: Conditions for preparation of polysomal poly(A) RNA, synthesis of [^{3}H]cDNA and hybridization with polysomal poly(A) RNA of PRL$^-$ strain have been described in Methods. [^{3}H]cDNA fractions were analyzed on 3% polyacrylamide gels using the system described by Loening (4) except that the samples were denatured in 50% formamide for 5 min at 70^o following quick cooling before application to the gel. Gels were analyzed by both staining and also by counting 2 mm slices after solubilizing in 30% H_2O_2 at 60^o. ●-●= [^{3}H]cDNA before hybridization; o-o= [^{3}H] cDNA, the unhybridized fraction obtained after complete reassociation with mRNA from PRL$^-$ strain, i.e. $cDNA_{PRL}$ fraction.

Fig. 2B. Alkaline sucrose gradient centrifugation analysis of $cDNA_{PRL}$: Alkaline sucrose gradient centrifugation analysis of [^{3}H] $cDNA_{PRL}$ is carried out by following the method described by Monahan et al (5). The [^{3}H] cDNA sample in 0.1M NaOH, 0.9M NaCl and 5 mM EDTA was layered on 6 ml of 8-18% sucrose in the same solution. The samples were then centrifuged in SW 50.1 rotor at 38000 rpm for 16 hr at 20^o in Beckman L3-50 ultracentrifuge. After centrifugation 5 drop fractions were collected and neutralized with 0.1M HCl and 0.5 ml dH_2O was added. Samples were counted in 10 ml of Aquasol. Samples of standard DNA markers such as synthetic polyDI (4.9s) and synthetic polyDG (6.7s) were analyzed under the similar conditions.

Fig. 2C. Polyacrylamide gel electrophoresis of poly(A) RNA: Procedure for the preparation of poly(A) RNA from PRL$^-$ strain and synthesis of matrix bound cDNA from it is described in Methods. Electrophoresis of [^{32}P] labelled RNA and (cont'd)

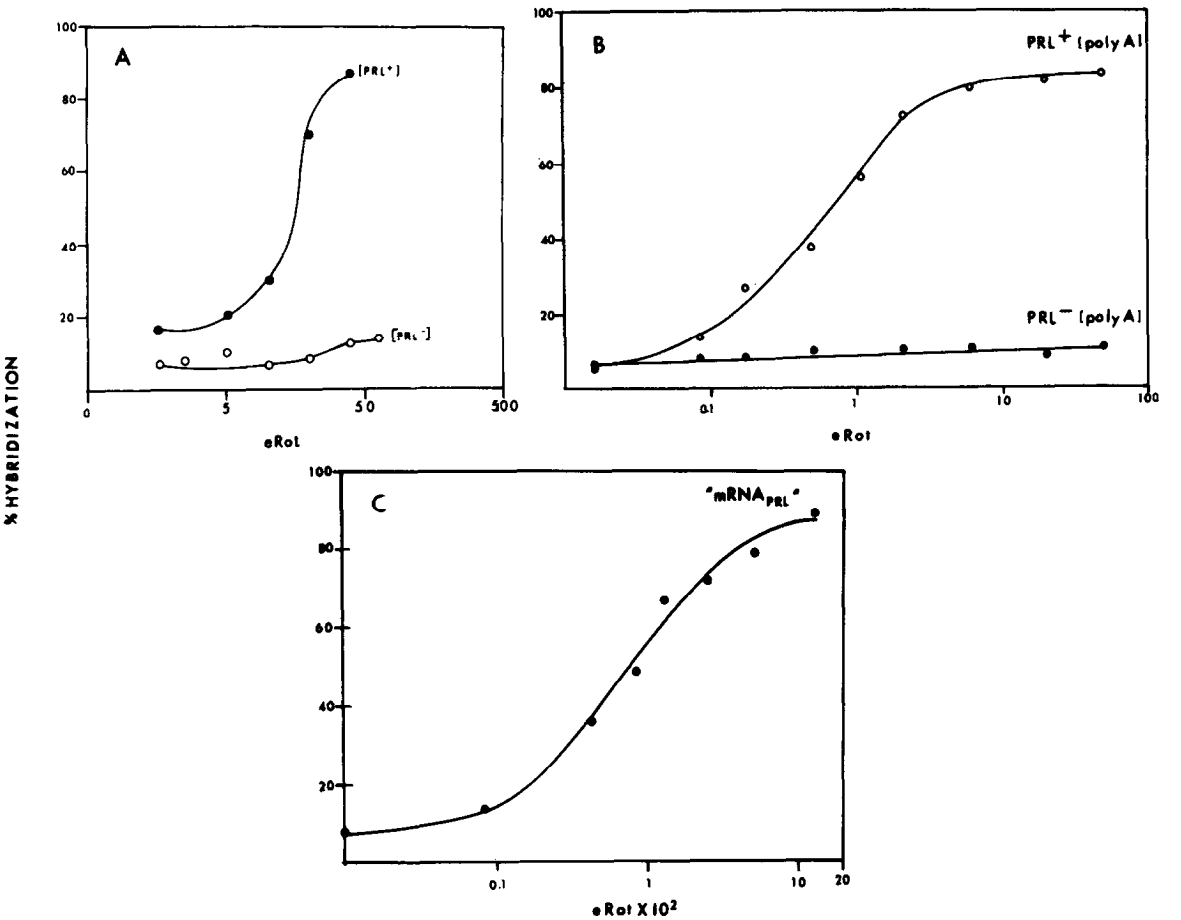

Fig. 3A. Reassociation kinetics of $cDNA_{PRL}$ with polysomal RNA: Preparation of $cDNA_{PRL}$ by following the scheme I of Fig. 1 is described in Methods. Hybridization was carried out in sealed plastic incubation tubes containing 1000 cpm, [^{3}H]cDNA, 10µg of total polysomal RNA, in hybridization buffer (0.1M HEPES, pH 7.0; 0.6M NaCl, 0.005M EDTA) in total volume of 25 µl. The reaction mixture is kept in a water bath at 100° for 2 min and then incubated in a 60° water bath for the desired period of time. After the incubation the reaction is terminated by freezing the samples in dry ice alcohol bath. The reaction mixture was then treated with S_1 nuclease to determine the extent of hybridization. S_1 nuclease resistant TCA precipitable radioactivity was determined (5). •-•= polysomal RNA from PRL$^+$ cells; o-o= polysomal RNA from PRL$^-$ cells.

Fig. 3B. Reassociation kinetics of $cDNA_{PRL}$ with polysomal poly(A) RNA: The preparation of [^{3}H]cDNA is described in Methods. Hybridization was carried out in sealed tubes containing 100 cpm of [^{3}H]cDNA and 1µg poly(A) RNA under the conditions described in Fig. 3A. After the incubation period the S_1

Fig.2C cont'd.

subsequent counting procedure is same as described in Fig. 2A. •-•= poly(A) RNA from polysomes of PRL$^+$ cells. o-o= The unhybridized fraction of the same RNA after removal of major fraction of total mRNA population by hybridization with matrix bound cDNA prepared from polysomal poly(A) RNA of PRL$^-$ cells. Arrows indicate the movement of the 4s and 18s RNA markers in the same gel system.

RNA (Fig. 3B, •-•), isolated from the PRL^- strain. Hybridization with polysomal poly(A) RNA of the PRL^- strain was allowed to occur as long as 96 hr. The $mRNA_{PRL}$ fraction (scheme II, Fig. 1) reassociated rapidly with $cDNA_{PRL}$ displaying an $eRot_{\frac{1}{2}}$ of 0.007-0.008 (Fig. 3C). This $eRot_{\frac{1}{2}}$ value is comparable to those obtained from studies carried out under similar hybridization conditions with other purified cDNAs and their complementary mRNAs such as $cDNA_{globin}$ /$mRNA_{globin}$ ($eRot_{\frac{1}{2}}$ 0.006) (5), thus suggesting that the $cDNA_{PRL}$ fraction is highly purified.

Results of hybridization experiments also suggest that the $mRNA_{PRL}$ fraction prepared according to the scheme II, Fig. 1 is greatly enriched for $mRNA_{PRL}$ sequences. Based on the predicted $eRot_{\frac{1}{2}}$ obtained by hybridization of a given cDNA to its purified mRNA, it can be estimated that at least 75% of the $mRNA_{PRL}$ enriched preparation contains $mRNA_{PRL}$ sequences. In comparison, $mRNA_{PRL}$ sequences comprise only 2% of the total polysomal poly(A) RNA of TRH treated cells. It is important to note that only translatable mRNA was utilized to prepare $cDNA_{PRL}$. Our previous results (2) have clearly demonstrated that the PRL^- cells do not contain any translatable $mRNA_{PRL}$. Although the major difference in the functional mRNA population of these two cell strains is $mRNA_{PRL}$, the PRL^+ cells may contain a small percentage of one or more species of mRNA aside from $mRNA_{PRL}$ which is/are not present in the total translatable mRNA of the PRL^- strain. The results presented in this report cannot rule out this possibility. Nonetheless, the $eRot_{\frac{1}{2}}$ value (0.007) observed when $cDNA_{PRL}$ is hybridized to the $mRNA_{PRL}$ enriched fraction and the mobility of the [^{3}H]$cDNA_{PRL}$ as a sharp band after polyacrylamide gel electrophoresis suggest that the $cDNA_{PRL}$ probe is highly purified.

To understand the mechanisms of action of different modulators of PRL syn-

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Figs.3B and 3C cont'd.
nuclease resistant TCA precipitable radioactivity was determined in order to quantitate the extent of hybridization. o-o= Hybridization with poly(A) RNA from PRL^+ cells. •-•= Hybridization with poly(A) RNA from PRL^- cells.

Fig. 3C. Reassociation kinetics of $cDNA_{PRL}$ with $mRNA_{PRL}$: The procedure for the preparation of $cDNA_{PRL}$ and the $mRNA_{PRL}$ fractions are described in Methods. All hybridization conditions and subsequent quantitation of S_1 nuclease resistant radioactivity are the same as described in legend to Fig. 3A. The amount of $mRNA_{PRL}$ fraction used per 25µl incubation is 50 ng.

thesis in GH-cells it is of considerable importance that a highly purified $cDNA_{PRL}$ probe is available. The ease of isolation of this $cDNA_{PRL}$ and its high degree of purity will make it an ideal starting material for amplification of the $cDNA_{PRL}$ by cloning.

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