Estrogen Withdrawal in Chick Oviduct. Evidence for Continued Expression of Active Unique Genes Using an "Expressed" DNA Probe†

Shigeki Mizuno‡ and Ronald F. Cox*

ABSTRACT: We have analyzed the effect of estrogen on the kinds of unique DNA sequences which are transcriptionally expressed in chick oviduct with an "expressed" DNA probe. Steady-state nRNA in estrogen-stimulated chick oviduct represents about 25% of the complexity of total chick unique DNA. To purify this expressed DNA fraction, chick unique DNA was isolated, nick-translated, and hybridized to chemically mercurated oviduct nRNA (Hg-nRNA); the resulting hybrids were bound to sulfhydryl-Sepharose, and DNA was selectively recovered by thermal elution in formamide buffer. To compare the sequence homology between nRNAs isolated from oviduct before or up to 6 days after estrogen withdrawal, trace amounts of expressed DNA derived from estrogen-stimulated oviduct were hybridized in RNA-excess reactions. All nRNAs hybridized with equal efficiency. Furthermore, hybridization of expressed DNA to nRNA mixtures showed that nRNA from nonwithdrawn and withdrawn oviduct contained a similar set of unique sequences. The data indicate that, at most, only a small percentage (0–5%) of transcriptionally active unique DNA sequences are shut down when estrogen is removed from the circulation.

An important, and currently equivocal, question relating to the action of estrogen in chick oviduct is whether, in a given target cell, potentially active genes are switched on and off in response to the hormone. The early data of Liarakos et al. (1973) suggested that a 28% increase (from 9 to 11.5%) in the fraction of chick DNA which was expressed occurred after prolonged estrogen stimulation. The analysis was complicated by the fact that, whereas estrogen-treated tissue consists primarily of tubular gland cells which synthesize egg white proteins (Oka & Schimke, 1969; Palmiter & Gutman, 1972), such cells are ostensibly absent in immature tissue (Cox & Sauerwein, 1970). Thus, mitogenic effects of the steroid and problems in comparison of RNAs derived from tissues containing mixtures of different cell types preclude a clear-cut interpretation. More recently, Monahan et al. (1976) analyzed the kinetic complexity of polyadenylated nRNAs[1] in estrogen-stimulated oviduct and tissue withdrawn from steroid treatment for 12 days. They concluded that RNA from the latter source was approximately 50% as complex as that from estrogen-stimulated tissue. However, since the results could be attributed to a hormone-dependent alteration in the spectrum of RNA sequences which are polyadenylated, this explanation is also open to question.

To analyze the problem further, we attempted to measure directly the sequence overlap between nRNAs from fully differentiated (estrogen stimulated) chick oviduct and from tissue that had been rapidly withdrawn from hormone treatment (for 1, 2, and 6 days), in order to minimize the influence of changing proportions of cell types on our results. While essentially taking the approach described by Liarakos et al. (1973), we were aware of the lack of sensitivity inherent in hybridization reactions containing total unique cellular DNA and excess nRNA since, in general, only a small fraction (perhaps 10%) of the DNA is driven into hybrid. We have therefore utilized the technique of nucleic acid mercuration (Dale et al., 1975) to enrich for "expressed" DNA in the unique chick DNA fraction and then used this preparation as a probe to compare nRNA populations. In this way, we increased the sensitivity of the hybridization assay seven- to eightfold. We show here that, within the limits of experimental error, the kinds of unique DNA sequences which are transcriptionally expressed in fully stimulated (nonwithdrawn) oviduct persist when the hormone is withdrawn from the circulation. Our results suggest that estrogens do not induce major alterations in the pattern of gene expression in fully differentiated target cells.

Materials and Methods

Animals and Hormone Treatment. Hexestrol pellets [(15 mg/pellet)/chick; Evans Medical Ltd., Liverpool, England] were implanted in 1-week-old White Leghorn pullets as described (Cox, 1977). After 3–5 weeks of implantation, oviducts or livers were removed (nonwithdrawn tissues). From other groups, intact hormone pellets were removed and oviducts were isolated after 1–6 days (withdrawn tissues). In other control experiments, male chicks were implanted with hexestrol pellets and nonwithdrawn livers were recovered.

Isolation and Labeling of Unique DNA. DNA was extracted and purified from livers of nonwithdrawn female chicks according to previously described methods (Mizuno & Macgregor, 1974), sheared by sonication to an average length of 350 nucleotides, and passed through a column of Chelex-100 (Bio-Rad, Richmond, CA) as described elsewhere (Mizuno et al., 1978). The sheared DNA was dissolved in 0.01 M Pipes (pH 6.7), 0.6 M NaCl, and 40% formamide, head-denatured in boiling water for 5 min, and incubated at 43 °C to D_0t (the product of the initial concentration of DNA nucleotides in moles per liter and the time of incubation in seconds) = 200 M s. The mixture was applied to a column of hydroxyapatite (Hypatite-C, Clarkson Chemical Co., Williamsport, PA), and a single-stranded DNA fraction was obtained as described (Mizuno & Macgregor, 1974; Mizuno et al., 1978). The

† From the Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota 55901. *Received August 29, 1978; revised manuscript received January 19, 1979.* This work was supported by National Institutes of Health Grant HD-9140-C and the Mayo Foundation.

‡ Present address: Department of Applied Biological Science, Science University of Tokyo, Noda City 278, Japan.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid); TN buffer, 0.01 M Tris-HCl (pH 7.5) and 0.1 M NaCl; TN-MSH buffer, TN buffer containing 0.1 M 2-mercaptoethanol; nRNA, nuclear RNA; Hg-RNA, mercurated RNA; mRNA, messenger RNA.

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single-stranded DNA fraction was dialyzed against water, lyophilized, and reincubated to D_0t = 4000 (same conditions as the previous incubation). The mixture was fractionated on hydroxyapatite, and the double-stranded DNA fraction obtained was dialyzed against water, lyophilized, and then labeled by "nick-translation" in vitro with *Escherichia coli* DNA polymerase I (Grade I, Boehringer Mannheim Biochemicals) and all four [^{3}H]deoxyribonucleoside triphosphates (ICN, Irvine, CA, and NEN, Boston, MA; 8–24 Ci/mmol) according to the conditions described (Macgregor & Mizuno, 1976). The labeled DNA was again reannealed to D_0t = 10 M s, and a single-stranded DNA fraction was obtained on hydroxyapatite. This fraction was dialyzed against water, lyophilized, and dissolved in water. The specific radioactivity of the labeled DNA was approximately 10^7 cpm/μg.

Preparation of nRNA. nRNA was prepared according to procedures described elsewhere (Cox, 1977; Mizuno et al., 1978). Briefly, chick oviduct nuclei were isolated and treated with 80 μg of DNase I/mL in the presence of 30 mM $MgCl_2$ at 21 °C for 25 min. Ammonium sulfate (330 mM final) was then added to lyse nuclei, and RNA was extracted with phenol–chloroform. The DNase I used was pretreated with agarose–5′-[(*p*-aminophenyl)phosphoryl]uridine 2′(3′)-phosphate (Miles-Yeda Ltd., Rehovoth, Israel) according to Brison & Chambon (1976). All aqueous solutions used for RNA extraction were treated with 0.05% diethyl pyrocarbonate and boiled. Considerable care was taken to remove DNA from the nRNA by including three steps of digestion with DNase I (Worthington Biochemical Corp., Freehold, NJ), heat denaturation (at 85 °C) in the presence of 50% formamide, and Sephadex G-200 column chromatography. To confirm that all hybridization reactions containing nRNA excess were driven only by RNA, nRNA samples were digested at 37 °C for 18 h in 0.3 N KOH, neutralized, and then hybridized for up to 20 days (R_0t = 5.2 × 10^4 M s) with trace amounts of labeled chick DNA. No hybridization above background levels (reactions containing yeast RNA instead of chick nRNA) was observed.

Mercuration of RNA in Vitro. The following conditions of mercuration were adopted from Dale et al. (1975), in which 10–15% of the total bases in RNA are expected to be mercurated. Reaction mixtures contained 1.5 mg of nonwithdrawn oviduct nRNA or yeast RNA (Sigma, Type XI; treated with 0.05% diethyl pyrocarbonate and boiled), 0.05 M sodium acetate (pH 6.5), and 36 μmol of mercuric acetate in a total volume of 8.0 mL. The mixture was incubated at 50 °C for 1.5 h and then passed through a column (2.7 × 32 cm) of Sephadex G-50 (fine) in 0.05 M sodium acetate (pH 6.5). The excluded mercurated RNA (Hg-RNA) was precipitated with 2.5 volumes of ethanol in the presence of 0.1 M sodium acetate (pH 6.5) at −20 °C, centrifuged, and dissolved in water. The Hg-RNA showed an absorption maximum at 271–273 nm and a minimum at 242 nm (in water). [^{3}H]nRNA (90 μg; 1000 cpm/μg), isolated from nonwithdrawn oviduct nuclei after in vitro incubation with [^{3}H]UTP under the conditions described (Mizuno et al., 1978), was also mercurated as above but in a smaller scale reaction.

Hybridization. Hybridization of ^{3}H-labeled unique DNA with excess, total, sheared DNA was carried out in one of two ways. (*1*) *Pipes–Formamide System.* Reaction mixtures (20 μL) containing 6000 cpm of [^{3}H]DNA, 45 μg of unlabeled DNA, 0.01 M Pipes (pH 6.7), 0.6 M NaCl, and 40% formamide were sealed in 50-μL glass capillaries (siliconized, treated with 0.05% diethyl pyrocarbonate, and boiled), heat-denaturated (95 °C for 5 min), and then incubated at 43 °C in a water bath. After incubation (20 s–7 days), mixtures were diluted in 4.0 mL of 0.08 M phosphate buffer (an equal volume mixture of 0.8 $NaH_2PO_4 \cdot H_2O$ and 0.08 M Na_2HPO_4, pH 6.7) and fractionated on a hydroxyapatite column (0.5 × 5.5 cm) at 60 °C into single- and double-stranded DNA (Mizuno & Macgregor, 1974; Mizuno et al., 1978). The extent of reassociation of unlabeled DNA was determined by absorbance at 260 nm, and hybridization of [^{3}H]DNA was determined from the radioactivity in each fraction. (*2*) *Hepes–Formamide System.* Reaction mixtures (5 μL) contained 1000–2000 cpm of [^{3}H]DNA, 25 μg of unlabeled chick DNA, 0.025 M Hepes (pH 6.8), 0.5 M NaCl, 0.5 mM EDTA, 0.1% $NaDodSO_4$, and 50% formamide and were sealed in a 10-μL glass capillary. Mixtures were heat-denatured (95 °C for 3 min) and then incubated at 43 °C. After incubation (15 s–15 days), mixtures were rinsed into 0.90 mL of S-1 buffer [0.03 M sodium acetate (pH 4.5), 0.075 M NaCl, 2 × 10^{-4} M $ZnSO_4$, 20% glycerol, and 20 μg/mL of calf thymus DNA (P-L Biochemicals, Milwaukee, WI)], and 0.20 mL of this mixture was removed for determination of total radioactivity. To the remainder, 0.01 mL (40 units in 0.05 M sodium acetate, pH 4.5) of S-1 nuclease (Sigma, Type III) was added, and the mixture was incubated at 45 °C for 30 min. Total and S-1 nuclease resistant radioactivity were determined after precipitating in 10% Cl_3AcOH in the presence of 50 μg of bovine serum albumin, collecting on glass-fiber filters (Whatman, Inc., Clifton, NJ; 2.4-cm diameter), washing with cold 5% Cl_3AcOH containing 3% sodium pyrophosphate, drying, and placing in Spectrafluor PPO–POPOP scintillation fluid (Amersham Corp., Arlington Heights, IL).

Hybridization of ^{3}H-labeled unique DNA with excess nRNA was carried out in the Hepes–formamide system. Reaction mixtures (2.5 μL) containing 1000 cpm of [^{3}H]DNA and 25–50 μg of unlabeled RNA were sealed in 5-μL glass capillaries. Reaction mixtures were heat-denatured and incubated for 1–24 days. Extent of hybridization was assayed with S-1 nuclease as described above. Control incubations (in which nRNA was replaced by an equal amount of yeast RNA) were run in parallel, and background radioactivity scored as S-1 resistant hybrid (ranging from 0 to 3.7% of the input counts per minute) was subtracted from experimental values.

To prepare expressed DNA, ^{3}H-labeled unique DNA was hybridized with Hg-RNA in the Hepes–formamide system, except that the buffer lacked EDTA and the reaction volume was increased to 0.1 mL. For these reactions, it was important to use freshly prepared Hg-nRNA. When a portion of Hg-nRNA used in the first cycle of hybridization was frozen 2–3 weeks and then used in the second hybridization, the level of hybrid obtained was reduced to less than 50% of that obtained with freshly mercurated RNA. This problem appears to be caused by a decrease in the solubility of Hg-nRNA as a function of storage time.

Sulfhydryl-Sepharose Chromatography. Sulfhydryl-Sepharose (SH-Sepharose) was prepared according to Dale et al. (1975) with Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) and had 1.9–3.2 μmol of SH group/mL of packed volume. SH-Sepharose was packed in a siliconized, water-jacketed glass column (inner diameter 1.2 cm) and washed with 40 mL of TN buffer [0.01 M Tris-HCl (pH 7.5) and 0.1 M NaCl] prior to application of samples. The quantity of SH-Sepharose was dependent on the amount of Hg-nRNA in the sample, but a column 1.2 × 4.0 cm provided excess SH groups for samples containing 1 mg of Hg-nRNA. Samples to be loaded were first diluted in TN

buffer (up to 6 mL), applied to the column at room temperature (21–23 °C), and usually recycled. Then the column was washed with 20 mL of TN buffer, followed by 20 mL of formamide buffer [0.01 M Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, and 60% formamide]. Next, the temperature of the column and the formamide buffer was raised to 65 °C, and the hybridized [^{3}H]DNA was dissociated and eluted with 30 mL of formamide buffer. Bound materials were eluted with approximately 20 mL of TN-MSH buffer (TN buffer containing 0.1 M 2-mercaptoethanol) after cooling the column to room temperature. Fractions [1.8 mL (60 drops)] were collected throughout the process, and aliquots were mixed with Aquasol (NEN) to determine radioactivity in a scintillation counter.

Recovery of Expressed DNA. Fractions containing ^{3}H-labeled unique DNA eluted from SH-Sepharose at 65 °C in formamide buffer were pooled, and 30 μg of alkali-treated, sheared, carrier calf thymus DNA was added. The mixture was loaded onto a Sephadex G-50 column (2.7 × 33 cm), equilibrated in H_2O. The excluded peak of radioactivity was pooled and lyophilized.

Alkali Treatment. To remove any Hg-nRNA contaminating the final expressed DNA fraction, the latter was incubated in 0.3 N NaOH and 1 mM EDTA at 95 °C in a water bath for 3 min, neutralized with HCl, passed through Sephadex G-50 (see above), and finally lyophilized.

Results

Isolation of Unique Chick DNA. To prepare expressed DNA it was first necessary to isolate unique (single copy) DNA. Recent studies on the kinetics of reassociation of total chick DNA (Mizuno et al., 1978) revealed the presence of two principal components: a minor one (about 5% of the genome; k = 1.23 M^{-1} s^{-1}) comprising repetitive sequences and a major one (about 80% of the genome; k = 5.20 × 10^{-3} M^{-1} s^{-1}) comprising unique sequences. To purify this latter fraction, total chick DNA was sheared to an average length of 350 nucleotides and subjected to two cycles of reassociation. In the first cycle, DNA was reannealed to D_0t = 200 M s, and the single-strand fraction was recovered on hydroxyapatite. This fraction was again reannealed to D_0t = 4000 M s, and double-stranded DNA was recovered and labeled by nick-translation. The latter was then reannealed to D_0t = 10 M s, and the single-stranded (unique) DNA was finally recovered on hydroxyapatite.

Reassociation profiles of unlabeled total and labeled, nick-translated unique DNA are compared in Figure 1, and the data were subjected to least-squares analysis with a computer program (Britten et al., 1974; Pearson et al., 1977). As indicated in the legend to Figure 1, values obtained for kinetic parameters defining total DNA components were similar to those measured previously (Mizuno et al., 1978). For purified, nick-translated, unique DNA, about 80% hybridized as a single component and had the typical kinetic behavior of single-copy DNA sequences (k = 4.14 × 10^{-3} M^{-1} s^{-1}). The rate of hybridization was about 20% slower than that shown by unlabeled DNA. This may be due to the presence of relatively shorter DNA strands since an analysis of DNA fragments on isokinetic sucrose gradients according to Noll (1967) revealed that the average length of DNA is reduced from 350 to approximately 290 nucleotides after nick-translation (results not shown).

Although middle-repetitive DNA appears to be effectively removed during purification, 7% of the unique DNA fraction behaved as double-stranded DNA at D_0t = 10^{-2} M s, suggesting contamination by rapidly reannealing "fold-back"

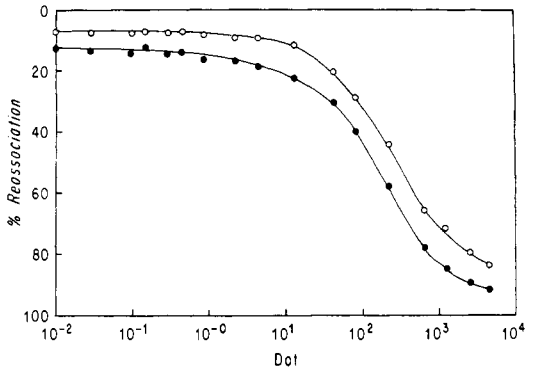


FIGURE 1: Hybridization of unique DNA with total, sheared, chick DNA. Reactions were carried out in Pipes–formamide, and the extent of reassociation was assayed by hydroxyapatite column chromatography. (●) Reassociation of unlabeled DNA; (○) hybridization of ^{3}H-labeled unique DNA. In each case, the continuous line represents the best fit obtained by computer analysis (see text). The computer fit resolved the following components having a second-order rate constant (k) and representing a fraction (%) of the input DNA: for unlabeled DNA, component 1 (k = 1.23 M^{-1} s^{-1}, 5.0%) and component 2 (k = 5.20 × 10^{-3} M^{-1} s^{-1}, 78%); for labeled unique DNA, component 1 (k = 4.14 × 10^{-3} M^{-1} s^{-1}, 78.1%).

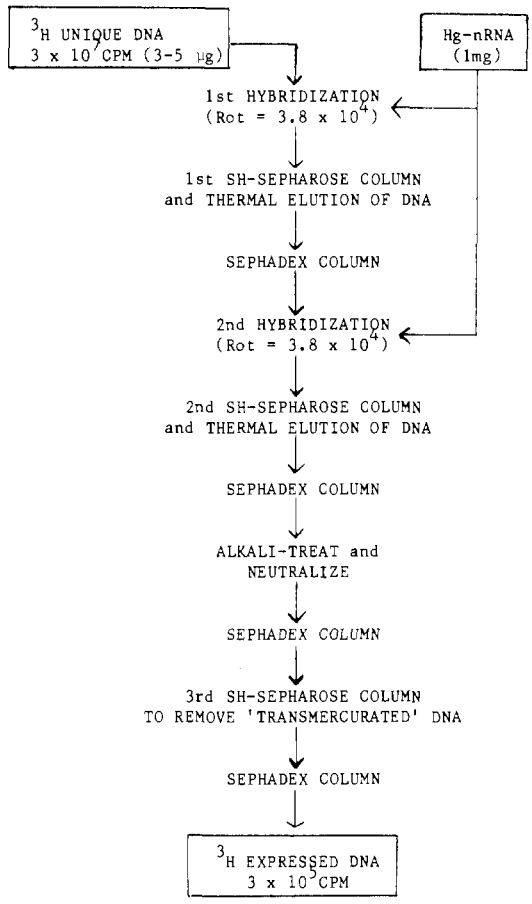
DNA (Angerer et al., 1975) or the occurrence of nonspecific binding of single-stranded DNA to hydroxyapatite. This component is considerably reduced when hybrids are assayed with S-1 nuclease (see Figure 4), indicating that the major fraction which binds to hydroxyapatite at low D_0t values is unique DNA. Based on these kinetic analyses, we estimate that the maximum level of contamination of our unique DNA preparation with "fold-back" DNA and middle-repetitive DNA is 1–2% in both cases.

Purification of Expressed DNA. An outline of the method employed to purify expressed DNA is shown in Scheme I. Briefly, nRNA was isolated from estrogen-stimulated (nonwithdrawn) chick oviduct and then chemically mercurated. Next, ^{3}H-labeled unique chick DNA was hybridized to this RNA (Hg-nRNA), and the resulting hybrids were recovered on SH-Sepharose. DNA in hybrid form was then eluted at 65 °C in the presence of buffer containing 60% formamide. In preliminary experiments, we found that digestion of DNA–Hg-nRNA hybrids with S-1 nuclease prior to SH-Sepharose chromatography considerably impaired the subsequent hybridizability of the DNA. We therefore omitted the digestion step and instead performed a second cycle of hybridization and chromatography.

The rationale for the recovery of purified expressed DNA from DNA–Hg-nRNA hybrids bound to SH-Sepharose was based on the results shown in Figure 2. We sought to establish conditions in which hybridized DNA could be removed, while retaining Hg-nRNA on the column. When ^{3}H-labeled unique DNA–Hg-nRNA hybrids were bound to SH-Sepharose and then washed with formamide buffer at increasing temperatures (22–90 °C), 98% of labeled DNA was eluted at 60 °C. On the other hand, Hg-nRNA (labeled for this experiment) was highly resistant to thermal elution; only 15% was released at 60 °C and 60% remained bound even at 90 °C. However, as expected, the remaining RNA could be released with 2-mercaptoethanol after cooling the column to room temperature.

In the first cycle of hybridization, unique DNA was hybridized essentially to completion (R_0t = 38 000 M s) with excess chick Hg-nRNA, and 5–6% of the input DNA was removed from SH-Sepharose by stepwise elution with formamide buffer at 65 °C. A number of nucleic acid mixtures were subjected to chromatography on SH-Sepharose to es-

Scheme I: Procedure for the Isolation of Expressed DNA



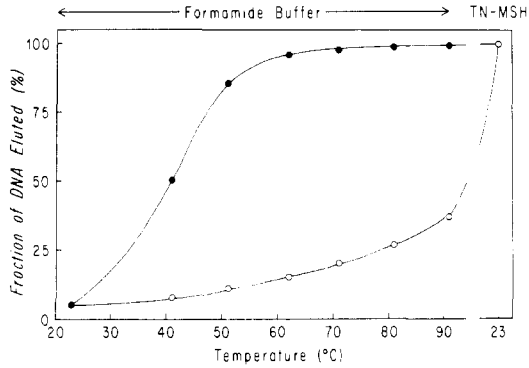


FIGURE 2: Thermal elution of mercurated RNA and hybridized DNA from SH-Sepharose. Nucleic acid samples were loaded onto SH-Sepharose columns (1.2 × 2.5 cm) in TN buffer and eluted in formamide buffer at increasing temperatures. The column was then cooled to 23 °C, and bound DNA was eluted with TN buffer containing 0.1 M 2-mercaptoethanol (TN-MSH). The distribution of labeled DNA in each fraction was then plotted. The following samples were analyzed. (●) A mixture of ^{3}H-labeled unique DNA (3.8 × 10^6 cpm) and nonwithdrawn oviduct Hg-nRNA (990 µg) was previously hybridized to R_0t = 36 600 M s. DNA–RNA hybrids were recovered after treatment with S-1 nuclease and chromatography on Sephadex G-50, and a portion of the hybrid (11 000 cpm) was loaded onto SH-Sepharose. (○) A sample of unhybridized ^{3}H Hg-nRNA (11 000 cpm) prepared as described under Materials and Methods.

timate background levels. When unique DNA (unannealed), unique DNA (reannealed to D_0t = 1 × 10^3 M s), a mixture of unique DNA and unlabeled oviduct Hg-nRNA (unhybridized), or mixtures of unique DNA and either mercurated yeast RNA or unmercurated chick oviduct nRNA (both hybridized to R_0t = 3.0 × 10^3 M s) were passed through SH-Sepharose, less than 0.25% of the radioactivity in loaded DNA was retained and subsequently eluted with formamide buffer (at 65 °C) in all cases.

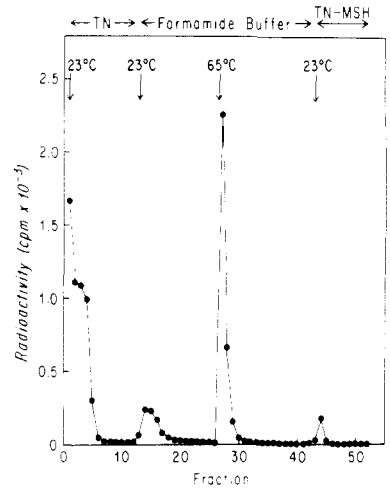


FIGURE 3: Thermal elution of purified expressed DNA from SH-Sepharose (second cycle). Purified expressed DNA, approximately 1.5 × 10^6 cpm, obtained from the first cycle of hybridization and affinity chromatography was rehybridized with 1.06 mg of nonwithdrawn oviduct Hg-nRNA in Hepes–formamide buffer. The mixture was loaded onto SH-Sepharose, and the pattern of elution of labeled DNA with TN buffer, formamide buffer, or TN-MSH buffer at either 23 or 65 °C (as indicated) was monitored. Hybridized expressed DNA which eluted at 65 °C in formamide buffer (fractions 27–29) was used in further purification steps.

A typical elution profile obtained after the second cycle of hybridization is shown in Figure 3; in this case, the percentage of input radioactivity recovered in the expressed DNA fraction was about 30%. DNA recovered was heated in alkali to degrade any remaining Hg-nRNA and again passed through SH-Sepharose (see Scheme I). At least 90% of the [^{3}H]DNA did not bind to the column, and this fraction was used as the expressed DNA preparation. Unexpectedly, a significant fraction of DNA (10%) did bind to the column compared to background levels (0.25%). This fraction probably contains Hg atoms covalently bound to DNA, resulting from a "transmercuration" reaction between Hg-nRNA and DNA during hybridization. This point was not confirmed, but, since background hybridization mixtures containing labeled DNA and mercurated yeast RNA did not result in enhanced, nonspecific binding of DNA to SH-Sepharose (see above), significant levels of transmercuration may only result when Hg-RNA and DNA bases are in close proximity (that is, in hybrid form). In any case, the final SH-Sepharose step removes these modified DNA strands. The recovery of unique DNA in the expressed fraction, after being subjected to all steps outlined in Scheme I, was about 1%.

Characterization of Expressed DNA. The rates of reannealing of purified expressed DNA and the original unique DNA with unlabeled total chick DNA are compared in Figure 4, after scoring hybrids with S-1 nuclease. In both cases, nearly all hybrid formation occurs after D_0t = 10 M s, demonstrating that expressed DNA retains its single-copy characteristics after purification. Expressed DNA hybridizes slightly faster than unique DNA, probably because more reactive sequences are selected during its preparation. Note that values for both the rate and extent of hybridization are about 30–50 and 50–60%, respectively, of those measured with newly nick-translated unique DNA (Figure 1). We consider that most of this time-dependent decrease in reactivity is a consequence of the nick-translation procedure (see Discussion). Whereas, in Figure 1, hybridization was started immediately after nick-translation, labeled DNAs hybridized in Figure 4 were both 2.5 months old with respect to nick-translation. In

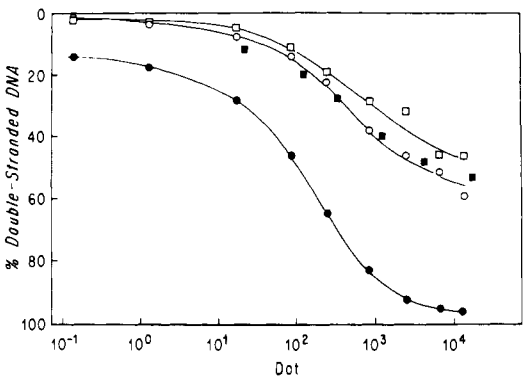


FIGURE 4: Reassociation of labeled DNA fractions with excess chick DNA. Samples (1000–2000 cpm) of ^{3}H-labeled expressed DNA (○), ^{3}H-labeled expressed DNA which was recovered in RNA–DNA hybrids after hybridization with excess nonwithdrawn nRNA (■), or ^{3}H-labeled unique DNA (□) were hybridized with sheared chick DNA (25 μg) in Hepes–formamide, and the rate of hybrid formation was scored with S-1 nuclease. Reassociation of unlabeled DNA (●) was assayed by hydroxyapatite column chromatography. Based on computer analysis of the rates of reassociation of the unique component of unlabeled DNA in the above buffer ($k = 3.87 \times 10^{-3}$ M^{-1} s^{-1}) and in Pipes–formamide ($k = 5.20 \times 10^{-3}$ M^{-1} s^{-1}), D_0t values on the abscissa were corrected (×3.87/5.2) to facilitate comparison of data in Figures 1 and 4.

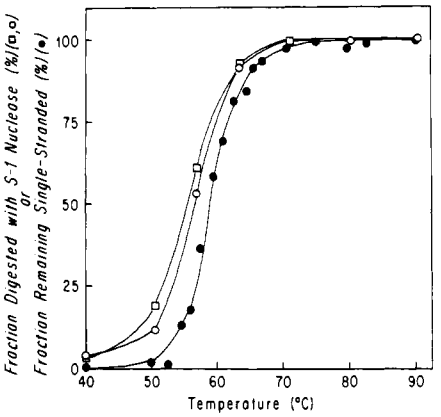


FIGURE 5: Thermal stability of hybrids between nick-translated DNA fractions and unlabeled chick DNA. Hybrids formed at $D_0t = 9 \times 10^3$ M s (under the conditions described in Figure 4) between unlabeled chick DNA and ^{3}H-labeled expressed DNA (○) or ^{3}H-labeled unique DNA (□) were incubated for 5 min at various temperatures in Hepes–formamide prior to determining S-1 nuclease resistant radioactivity. Native DNA (●) was sheared to an average size of 400 nucleotide pairs by sonication in 0.12 M phosphate buffer, purified on a hydroxyapatite column, and heat-denatured as described above, and the fraction of single-stranded DNA produced was determined with S-1 nuclease.

this case, the lower values are also caused partly by the different assay methods employed (hydroxyapatite vs. S-1 nuclease), since nuclease treatment generates comparatively "delayed" kinetic curves (Smith et al., 1975). But we observe similar differences when identical assays are used to monitor reassociation.

The thermal stability of the DNA–DNA hybrids analyzed in Figure 4 (hybridized to $D_0t = 9 \times 10^3$ M s) are compared in Figure 5. Hybrids formed between expressed DNA or unique DNA and unlabeled DNA exhibit similar T_m values (55–56 °C), which are 3–4 °C lower than the T_m of native, sheared chick DNA under our annealing conditions. Taken at face value, this indicates that nick-translated DNAs are 2–3% mismatched (Ullman & McCarthy, 1973). But, considering the relatively short average chain length of nick-translated DNAs, the fidelity of base pairing is probably closer to the native state.

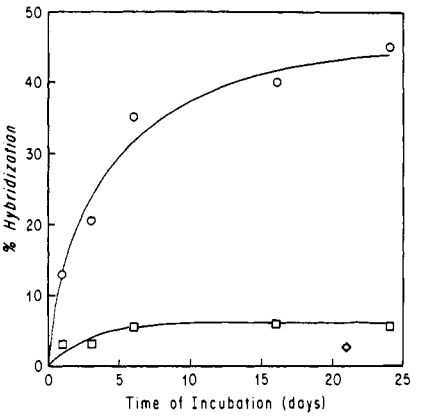


FIGURE 6: Hybridization of DNA fractions with excess oviduct nRNA. Samples (1000–2000 cpm) of ^{3}H-labeled expressed DNA (○) or ^{3}H-labeled unique DNA (□) were hybridized with excess, nonwithdrawn chick oviduct nRNA (25 μg) in Hepes–formamide (2.5 μL) for up to 24 days, equivalent to a R_0t value of 63 360 M s. The fraction of DNA resistant to S-1 nuclease was then plotted after subtracting background levels. For the latter, an equivalent amount of yeast RNA replaced nRNA, and a typical result obtained after S-1 digestion is indicated (◇).

In Figure 6, the fraction of unique and expressed DNAs which hybridized with nonwithdrawn chick oviduct nRNA was determined in RNA-excess reactions incubated to $R_0t = 6.3 \times 10^4$ M s, at which point complete saturation of complementary DNA was achieved. As shown, about 5% of unique DNA and 45% of expressed DNA were rendered nuclease resistant. To assess the apparent purity of expressed DNA on the basis of these results, a number of factors must be considered. First, the results in Figure 4 indicate that the percentage of hybridizable sequences in total unique and expressed DNA preparations is 50 and 55%, respectively. Using these estimates to correct for the plateau levels in Figure 6, we predict that 10% (5 × 100/50) of total unique DNA and 82% (45 × 100/55) of expressed DNA would be driven into hybrid if all DNA strands were hybridizable. Second, 50% of the labeled total unique DNA preparation consists of nonsense strand. Therefore, hybridization of 10% of unique DNA to nRNA (which only drives sense-strand annealing) represents 20% hybridization of the sense-strand unique DNA. When viewed in this light, we have achieved a 4.1-fold enrichment (82/20) of expressed DNA sequences relative to starting unique sense-strand DNA (compared to a theoretical purification of 5) or an 8.2-fold enrichment relative to total unique DNA.

To monitor the extent to which expressed DNA self-annealed in RNA-excess reactions, nRNA was replaced by yeast RNA. A typical result is plotted in Figure 4, and, in general, no more than 4% of expressed DNA was rendered S-1 resistant in these mixtures. In subsequent experiments, self-annealing controls were run in parallel and subtracted (see Materials and Methods).

Finally, the nature of the DNA sequences which actually hybridized with nRNA was checked. Expressed DNA was hybridized to completion and RNA–DNA hybrids were isolated by hydroxyapatite chromatography. After treatment with alkali to destroy RNA, DNA remaining was back-hybridized to unlabeled total chick DNA. As shown in Figure 4, the kinetics of reassociation was almost identical with that of the original expressed DNA.

Complexity of RNA. Experiments similar to that shown in Figure 6, in which total unique DNA was hybridized with excess nonwithdrawn chick oviduct nRNA, were performed for two different DNAs and several different RNA prepa-

Table I: Saturation Hybridization of Total Unique Chick DNA by Nonwithdrawn Chick Oviduct nRNA

DNA prepn	R_0t value (mol s L^{-1})	fraction of labeled DNA in hybrid form (%)
1	6.3×10^4	5.8[a]
2	4.7×10^4	5.0, 6.5, 7.6

[a] Each value was obtained with a different preparation of nonwithdrawn nRNA.

rations, as shown in Table I. The average value for the fraction of DNA hybridized at completion is 6.22%, which represents 12.44% of the sense-strand DNA (see above). Assuming that only 50% of both unique DNA preparations is hybridizable (see Figure 4), our best estimate for the percentage of unique sense-strand DNA which is expressed as steady-state nRNA in nonwithdrawn chick oviduct is 24.9. Since 78% of chick DNA is unique (Mizuno et al., 1978), this value represents transcriptional expression of 19% of the total sense-strand DNA complement in chick.

It is instructive to estimate the fraction of nRNA which drives the reaction with expressed DNA. Using the calculation derived by Hough et al. (1975) and comparing the rate of hybridization in Figure 6 with that of a kinetic standard (*E. coli* DNA) which was reannealed under identical conditions (Mizuno et al., 1978), we calculate that less than 1.5% of the mass of nRNA sequences drives the reaction. No doubt the major, nonhybridizable fraction consists of rRNA. In addition to indicating that the high complexity fraction of nRNA is present in very low abundance, this calculation serves to emphasize the importance of removing all traces of DNA from nRNA preparations (see Materials and Methods) before initiating studies of this nature.

Hybridizing Expressed DNA with Various nRNA Preparations. The expressed DNA fraction purified here was complementary to the steady-state nRNA population present in chick tissue under the full influence of estrogen. To examine the effect of acute estrogen withdrawal on the extent of unique DNA expression, trace amounts of expressed DNA were hybridized to completion with oviduct nRNAs isolated before withdrawal (nonwithdrawn) or after 1, 2, and 6 days of withdrawal, and the results are shown in Figure 7. Unexpectedly, RNA from all sources saturated expressed DNA to very similar levels; that is, 44–48% of the DNA probe was hybridized at the plateau (R_0t = 63 000 M s). To strengthen the conclusion that hybridizable DNA was fully saturated at this R_0t value, twice as much nRNA from nonwithdrawn and 2-day withdrawn tissue was hybridized with DNA; as shown, the plateau level attained was the same.

The data in Figure 7 clearly indicate that various nRNAs can saturate a similar fraction of expressed DNA, but it was necessary to establish whether in each case the fraction hybridized contained the same DNA sequences. For this, mixing experiments were performed, with a single source of expressed DNA and two independent preparations of each nRNA. As shown in Table II, within the limits of experimental error, we could detect no increase in the fraction of expressed DNA driven into hybrid when mixed nRNA samples were used compared to nonwithdrawn nRNA alone, indicating that all withdrawn nRNA samples tested contained the same spectrum of unique RNA sequences as was found in nonwithdrawn nuclear RNA.

When nonwithdrawn oviduct expressed DNA was challenged with nRNAs from other chick tissues, relatively less DNA probe was rendered double stranded. As shown in Figure 7, male or female liver nRNAs saturated DNA to 70 and 80% of the level seen with oviduct nRNA. These results concur with observations made by other investigators (Young et al., 1976; Axel et al., 1976; Ryffel & McCarthy, 1975), indicating a high degree of homology between RNAs expressed in different tissues of the same animal. However, when guinea pig liver nRNA was used as driver, only a small fraction of expressed DNA hybridized.

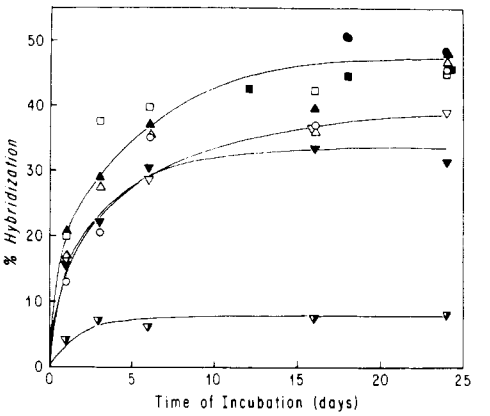


FIGURE 7: Hybridization of expressed DNA with various nRNA preparations. Samples of ^{3}H-labeled expressed DNA (1000 cpm) were hybridized with excess nRNA (25 μg) from nonwithdrawn oviduct (○), 1-day-withdrawn oviduct (△), 2-day-withdrawn oviduct (□), 6-day-withdrawn oviduct (▲), nonwithdrawn female liver (▽), nonwithdrawn male liver (▼), or guinea pig liver (▽) in Hepes–formamide (2.5 μL), and hybrid formation was assayed with S-1 nuclease. The R_0t value attained after 24 days of incubation was 63 360 M s. Hybridization was also carried out in the presence of twice as much nRNA (50 μg) from nonwithdrawn oviduct (●) or 2-day-withdrawn oviduct (■). For convenience, these points are plotted at incubation times equivalent to half the actual R_0t value attained.

Table II: Saturation of Nonwithdrawn Expressed DNA by nRNA Mixtures

expt[a]	source of nRNA	fraction of DNA hybridized[b] (%)
1	nonwithdrawn	50
	nonwithdrawn + 2-day withdrawn	48
	nonwithdrawn + 6-day withdrawn	50
2	nonwithdrawn	53
	nonwithdrawn + 2-day withdrawn	47
	nonwithdrawn + 6-day withdrawn	48

[a] Different batches of nRNA (from the tissue sources indicated) were used in experiments 1 and 2. [b] Hybridization was performed as described in the legend to Figure 7, with 50 μg of nonwithdrawn RNA or 25 μg of each RNA in "mixed" nRNA experiments. The fraction of DNA scored as hybrid was determined after hybridization to a R_0t value of 5.05×10^4 M s. All nRNA batches prepared from withdrawn oviduct were capable of driving at least 45% of expressed DNA into S-1 nuclease resistant hybrids.

Discussion

DNA probes complementary to cellular RNA represent an important tool in the analysis of gene expression. In cases where, for various reasons, reverse transcription of an RNA fraction is unsuitable, DNA probes have been obtained by hybridizing total genomic DNA with an excess of purified RNA and then isolating hybrids by nuclease digestion and hydroxyapatite chromatography (Galau et al., 1976). Unfortunately, since DNA contains both sense and nonsense strands, DNA reannealing also occurs in such reactions. This problem (a) limits the amount of DNA one can use in the hybridization mixture, (b) complicates the determination of

true plateau levels of RNA–DNA reannealing, and (c) precludes a clean separation of RNA–DNA from DNA on hydroxyapatite, since DNA–DNA hybrids also bind. Separation of RNA–DNA duplex from DNA has been attempted with equilibrium density centrifugation, but some overlap of these species on the gradient does occur (Hames & Perry, 1977).

Recent developments in the technique of nucleic acid mercuration and recovery of products on sulfhydryl-agarose supports (Dale et al., 1975; Dale & Ward, 1975) suggested to us a promising way to avoid these difficulties. The technique we describe, in which unique DNA is hybridized with mercurated RNA and hybrids are recovered on SH-Sepharose, has certain advantages. First, reannealed DNA–DNA hybrids are effectively removed at the column step. Second, since DNA reannealing no longer presents a problem, the DNA input (relative to RNA input) can be increased, thus improving the yield of hybridized DNA. This might be a particularly useful feature for preparing expressed repetitive DNA sequences. Third, reactions can be easily scaled up since the purification process only involves a series of hybridization and column chromatography steps.

The principal difficulty we encountered relates to the technique of nick-translation. Rigby et al. (1977) have shown that label is randomly incorporated into DNA, and such products have been used successfully in both molecular and cytological hybridizations (Galau et al., 1976; Macgregor & Mizuno, 1976). In our hands, labeling DNA to high specific activity with tritium resulted in a gradual loss of hybridizability of DNA during the course of the experiments, and we suggest that the low yield (30%) of expressed DNA in the second purification cycle is due largely to this phenomenon. It seems to occur whether all four DNA precursors (our case) or only one (Galau et al., 1976) is used, and the latter authors have evidence that loss of reactivity is caused by strand scissions in the DNA.

The final expressed DNA fraction contained essentially unique sequences, reassociated with native DNA with good fidelity, and did not self-anneal to any significant extent (0–4%). The latter observation makes it unlikely that the DNA moiety which hybridized to Hg-nRNA during the purification consisted of "networks" of plus and minus DNA strands. Theoretically, we did not expect to achieve 100% purity with respect to expressed DNA because DNA–Hg-nRNA hybrids were not treated with S-1 nuclease, and small stretches of contiguous nonexpressed sense-strand DNA will copurify with the expressed fraction. Note that DNA was sheared to a small average strand length to minimize this problem. We estimate that expressed DNA is at least 80% pure with respect to sequences complementary to nonwithdrawn chick oviduct nRNA isolated under steady-state conditions.

One purpose of this work was to estimate the complexity of unique sequences in nRNA, but problems encountered in evaluating the hybridizability of nick-translated unique DNA preclude us from making a definitive statement. Our best estimate is that 25% of the unique sense-strand DNA is expressed as steady-state nRNA in nonwithdrawn oviduct. By way of comparison, Liarakos et al. (1973) calculated a value of 18% for the same parameter in nonwithdrawn chicks (injected with diethylstilbestrol). These authors did not correct for any loss of hybridizability of their unique DNA, although they labeled DNA in vivo. By synthesizing complementary DNA with reverse transcriptase and then back-hybridizing the DNA product to the original template, it has been estimated that polyadenylated nRNA and polyadenylated cytoplasmic mRNA from nonwithdrawn oviduct represent 4.5% (Monahan et al., 1976) and 0.65% (Cox, 1977) of unique sequence DNA, respectively. Thus, taking the value of 25% for the overall unique sequence expressed, it appears that approximately 20% of the total complexity of the RNA product becomes polyadenylated and 3% or less reaches the cytoplasm as polyadenylated mRNA.

The principal conclusion derived from the present work relates to the effect of estrogen on the kinds of unique DNA sequences expressed in chick oviduct. Taken at face value, the results show that of the high complexity nRNA species being expressed in estrogen-stimulated oviduct, only a small percentage, if any, are eliminated from the nRNA population up to 6 days after withdrawal of estrogen. Certain provisos must accompany such a statement. Firstly, given the margin of experimental error in our hybridization assay, we would be unable to detect a small decrease (1–5%) in the complexity of nRNA present in withdrawn tissue. But even this small fraction could represent the transcriptional inactivation of a considerable number of unique structural gene regions. Secondly, we cannot necessarily equate the presence of a given nRNA species after withdrawal with continued transcription of this species. Nevertheless, based on estimates of the average half-life of hnRNA, which range from 23 min in mouse L cells (Brandhorst & McConkey, 1974) to 3 min in HeLa cells (Soeiro et al., 1968), it is highly probable that persistence of chick oviduct nRNA sequences after withdrawal for 1–6 days reflects continued transcription of those species. Thirdly, given the design of the experiments, we have not addressed the question of whether new single-copy genes are expressed after withdrawal, but we think this unlikely for the following reason. Our expressed DNA preparation was undoubtedly contaminated with sequences not expressed in the estrogen-stimulated state, yet mixtures of withdrawn and nonwithdrawn nRNAs did not increase the extent to which expressed DNA hybridized. Fourthly, we have only analyzed the steady-state population of nRNA. Any extremely unstable RNA transcripts (whether regulated by estrogen or not) would remain undetected in this analysis.

In contrast to our findings, Monahan et al. (1976) claim that estrogen withdrawal (for 12 days) results in a dramatic decrease in the extent of gene expression in chick oviduct. Although we have not examined the effect of prolonged withdrawal, we question these conclusions on the following grounds. Firstly, since only polyadenylated RNA species were studied, their results could be explained by a change in the kinds of RNA templates being polyadenylated in response to withdrawal. Secondly, RNA fractions and their respective complementary DNAs were not cross-hybridized in order to directly determine their homology. Thirdly, comparison of the rates of back-hybridization of each complementary DNA to its own template is subject to problems of (a) differential contamination of template fractions by ribosomal RNA and (b) defining the completion of the reaction. With regard to the latter, Kleiman et al. (1977) show that accurate estimates of the base sequence complexity of low-abundance RNA species are difficult when this approach is used and considerably less sensitive than the saturation method used here.

Our present results extend and strengthen those obtained recently (Mizuno et al., 1978) while characterizing nRNA transcripts synthesized in isolated chick oviduct nuclei. We showed that labeled RNA synthesized in nuclei from nonwithdrawn chicks could be competed out equally well for complementary sequences on chick unique DNA by excess

amounts of nRNA from either nonwithdrawn or 2-day-withdrawn chick oviduct; similar results were obtained in crossover experiments with transcripts synthesized from 2-day-withdrawn nuclei. It is clear that a very early effect (within 48 h) of estrogen withdrawal in chick oviduct is a rapid depletion of ovalbumin mRNA levels in all major cell compartments (Cox, 1977). Additional data (Palmiter & Carey, 1974; Cox, 1977) indicate that estrogen withdrawal reduced the stability of ovalbumin mRNA. Consequently, we currently favor a model in which the essential action of estrogen at the level of gene expression in differentiated cells is to modify the rate of transcription and the stability of certain gene products but not the spectrum of unique structural genes which are available for transcription. A similar pattern of control may well exist in other systems, such as the sea urchin, where, in the developmental transition from the blastula to the pluteus state, large changes in the abundance of specific mRNA classes are seen (Galau et al., 1976) in the absence of alterations in the kinds of unique genes being expressed (Kleene & Humphreys, 1977).

Acknowledgments

We thank Nancy Tallman and Jean McFarland for their excellent technical assistance, Dr. T. Quinlan for help with the isokinetic gradients, and Dr. T. Ono for his critical comments.

References

Angerer, R. C., Davidson, E. H., & Britten, R. J. (1975) *Cell 6*, 29–39.

Axel, R., Feigelson, P., & Schutz, G. (1976) *Cell 7*, 247–254.

Brandhorst, B. P., & McConkey, E. H. (1974) *J. Mol. Biol. 85*, 451–463.

Brison, O., & Chambon, P. (1976) *Anal. Biochem. 75*, 402–409.

Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29E*, 363–418.

Cox, R. F. (1977) *Biochemistry 16*, 3433–3443.

Cox, R. F., & Sauerwein, H. (1970) *Exp. Cell Res. 61*, 79–90.

Dale, R. M. K., & Ward, D. C. (1975) *Biochemistry 14*, 2458–2469.

Dale, R. M. K., Martin, E., Livingston, C. C., & Ward, D. C. (1975) *Biochemistry 14*, 2447–2457.

Galau, G. A., Klein, W. H., Davis, M. M., Wold, B. J., Britten, R. J., & Davidson, E. H. (1976) *Cell 7*, 487–505.

Hames, B. D., & Perry, R. P. (1977) *J. Mol. Biol. 109*, 437–453.

Hough, B. R., Smith, M. J., Britten, R. J., & Davison, E. H. (1975) *Cell 5*, 291–299.

Kleene, K. C., & Humphreys, T. (1977) *Cell 12*, 143–155.

Kleiman, L., Birnie, G. D., Young, B. D., & Paul, J. (1977) *Biochemistry 16*, 1218–1223.

Liarakos, C. D., Rosen, J. M., & O'Malley, B. W. (1973) *Biochemistry 12*, 2809–2816.

Macgregor, H. C., & Mizuno, S. (1976) *Chromosoma 54*, 15–25.

Mizuno, S., & Macgregor, H. C. (1974) *Chromosoma 48*, 239–296.

Mizuno, S., Tallman, N. A., & Cox, R. F. (1978) *Biochim. Biophys. Acta 520*, 184–202.

Monahan, J., Harris, S. E., & O'Malley, B. W. (1976) *J. Biol. Chem. 251*, 3738–3748.

Noll, H. (1967) *Nature (London) 215*, 360–363.

Oka, T., & Schimke, R. T. (1969) *J. Cell Biol. 43*, 123–137.

Palmiter, R. D., & Gutman, G. A. (1972) *J. Biol. Chem. 247*, 6459–6461.

Palmiter, R. D., & Carey, N. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2357–2361.

Pearson, W. R., Davidson, E. H., & Britten, R. J. (1977) *Nucleic Acids Res. 4*, 1727–1737.

Rigby, P. W., Dieckmann, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.

Ryffel, G. U., & McCarthy, G. J. (1975) *Biochemistry 14*, 1379–1385.

Smith, M. J., Britten, R. J., & Davidson, E. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4805–4809.

Soeiro, R., Vaughan, M. Y., Warner, J. R., & Darnell, J. E. (1968) *J. Cell Biol. 39*, 112–118.

Ullman, J. S., & McCarthy, B. J. (1973) *Biochim. Biophys. Acta 294*, 416–424.

Young, B. D., Birnie, G. D., & Paul, J. (1976) *Biochemistry 15*, 2823–2829.