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Amplification and Evolution of Deoxyribonucleic Acid Sequences Expressed as Ribonucleic Acid†

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ABSTRACT: DNA sequences expressed as RNA have been isolated by utilizing nucleic acid hybridization techniques and RNA from various tissues of the cow. These expressed sequences have been used in an attempt to detect "gene amplification" in differentiated tissues. Large scale amplification of these transcribed DNA sequences can be ruled out. Evolutionary studies on these expressed DNA sequences indicate that they diverge at the same average rate as the bulk of the cow DNA.

Recent applications of DNA–RNA hybridization techniques have enabled us to isolate from several tissues nonrepeated DNA sequences which have been expressed by being transcribed into RNA. These isolated, expressed nonrepeated DNA sequences[1] (E⁻DNA) have been utilized in an attempt to detect gene amplification. As used here, amplification denotes a situation where a DNA sequence is present more frequently in a specific tissue or cell type than in a sperm cell or a different tissue or cell type.

It has been postulated that specific gene amplification plays a definite role in development. The best known examples of this phenomenon have been reported in oocytes of a number of species (Brown and Dawid, 1968) where the genes coding for ribosomal RNA appear to be preferentially amplified, the average number of rRNA genes found in premeiotic oocyte nuclei being as much as 1000 times the number found in somatic nuclei. Differential replication has been shown to exist on a larger scale in the polytene salivary chromosomes of *Drosophila hydei* (Dickson *et al.*, 1971). Here the nonrepeated sequences appear to be more highly amplified than repeated sequences. Other evidence suggests that gene amplification may occur routinely in other diptera. For example, Pavan (1965a) has observed in several species that incorporation of [^{3}H]thymidine into nonreplicating polytene chromosomes occurs in practically all bands, varying in extent from band to band. The chromosomal location of the additional DNA synthesis varies with the developmental stage of the organism and is associated with certain, but not all, chromosome puffs. Pavan postulates that chromosome differentiation may be a manifestation of the additional synthesis and accumulation of specific genes (1965a,b).

Tartof (1971) recently has described a case of apparent compensation for a sex-linked decrease in the number of rRNA genes. In *Drosophila* the multiple rRNA genes are found in the nucleolus organizer region of the X chromosome. In situations in which X0 males are produced, the male flies are found to have approximately 150 more ribosomal RNA genes per X chromosome than found in the XX females. When only one sex chromosome is present, a biochemical attempt appears to be made to compensate for the loss of ribosomal RNA genes. This compensation effect may be an extension of the magnification effect described by Ritossa (1968) and also appears to be a direct amplification of the ribosomal RNA genes.

Experimental Procedure

[^{3}H]Thymidine labeled DNA was isolated by the urea phosphate method (Britten and Smith, 1970) from calf kidney tissue culture cells. Nonrepeated DNA was isolated by denaturing the sheared DNA and then incubating this DNA to a C_0t[1] of 50 in 0.14 M phosphate buffer at 60°. The DNA was then passed over hydroxylapatite (Kohne, 1968; Kohne and Britten, 1972) where the single-stranded (nonrepeated) DNA was separated from the double-stranded (repeated) DNA.

Large amounts of the unlabeled tissues were powdered with Dry Ice in a blender. The powdered tissue was thoroughly mixed and stored at −70° as a single source of DNA and

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[1] Abbreviations used are: E-DNA, expressed or transcribed DNA sequence; C_0t, one-half the optical density of the nucleic acid at 260 mμ × hours of incubation.

RNA. Unlabeled DNA was isolated as previously described (Kohne, 1968) except that after ethanol precipitation the DNA was collected by centrifugation in order to collect both single- and double-stranded DNA. For RNA isolation, 40 g of tissue powder was blender homogenized in 130 ml of lysing solution (0.3 M NaCl–0.05 M Tris (pH 7.8)–0.01 M EDTA (pH 8.0), saturated with diethyl oxydiformate). The cells then were lysed by adding sodium dodecyl sulfate and Sarkosyl both to 0.5% final concentration. The solution was then blended in a sealed container to reduce viscosity. An equal volume of hot (60°) phenol was added and the mixture was incubated at 60° for 5 min with frequent agitation, cooled, and centrifuged to separate phases. The aqueous phase was kept at 0° while the interface was reextracted with 50 ml of the lysing solution, shaken with hot phenol (which contained 0.1% 8-hydroxyquinoline), cooled, and spun. The combined aqueous phases were shaken with an equal volume of a 50% chloroform–50% phenol mixture and centrifuged. Finally the aqueous phase was shaken again with an equal volume of chloroform, centrifuged, and aspirated for 10–15 min to remove traces of chloroform. $MgCl_2$ was added to 0.05 M and DNase to 10 μg/ml. Following incubation of the solution for 1 hr at room temperature, 5 ml of 0.25 M EDTA/100 ml of solution was added and the mixture was shaken once with chloroform–phenol and once with chloroform alone. The aqueous phase was then precipitated with 2 vol of cold (−20°) EtOH and stored at −20° for at least 4 hr. The precipitate was collected by centrifugation and dissolved in 0.01 M Tris, brought to 0.01 M $MgCl_2$, and again treated with 10 μg of DNase/ml of solution for 1 hr at 37°. The solution was brought to 0.015 M EDTA and shaken first with chloroform–phenol and then chloroform. The RNA in the aqueous phase was precipitated twice from 0.2 M NaCl, dialyzed overnight against 6 l. of 10^{-3} M phosphate buffer (phosphate buffer is composed of equimolar quantities of Na_2HPO_4 and NaH_2PO_4; the indicated molarity is for the PO_4) 10^{-4} M EDTA, and centrifuged for 30 min at 34,000 rpm in the 40 rotor of a Beckman Model L to sediment glycogen. RNA samples from the supernatants were assayed for DNA contamination by complete base hydrolysis of aliquots of RNA, followed by attempted hybridization of the neutralized hydrolysate with ^{3}H-repeated DNA from calf kidney cells. Purified RNA preparations were stored at −20° until use.

All DNA samples were sheared to the uniform single-stranded piece size of about 300–500 nucleotides by passing twice thorough a valve with a pressure drop of 50,000 psi. Fractionation and kinetic analysis were done with hydroxylapatite as described previously (Kohne, 1968). The useful capacity of the hydroxylapatite (Bio-Rad, batch no. 9149) was 1 mg of RNA/ml of packed bed. RNA in excess of this amount competed for column sites with DNA–RNA hybrids. Frozen cow tissues were obtained from the St. Louis Serum Co. and stored at −70°.

The technique used to isolate E-DNA sequences involves incubating a small amount of radioactive nonrepeated DNA with large amounts of unlabeled RNA from a specific source under conditions such that DNA–DNA duplexes form very slowly, while DNA–RNA duplexes form rapidly. The resulting double-stranded nucleic acids are separated from unreacted single-stranded DNA by hydroxylapatite fractionation. Under the conditions used, RNase treatment did not affect binding to hydroxylapatite of either double- or single-stranded DNA. The sequences in the DNA–RNA hybrids are collected by treating the hybrids with RNase and passing the mixture over hydroxylapatite under conditions allowing DNA–DNA hybrids to stick to the column, while the now single-stranded E-DNA sequences pass through. The E-DNA is then purified by deproteinization. The basic technique is described below in detail.

(1) Mix a high concentration (a final concentration of 5–50 mg/ml) of unlabeled RNA with a low concentration (0.1–10 μg/ml) of nonrepeated [^{3}H]DNA; denature by heating the sample to 100° and incubate in 0.48 M phosphate buffer at 66° for 4–6 days. The reaction mixture volume was typically 1 ml. (2) At the end of the incubation adjust the mixture to 0.14 M phosphate buffer–0.4% sodium dodecyl sulfate, 60°. Care should be taken to use a sufficient amount of hydroxylapatite to bind all the double-stranded nucleic acid. The material adsorbed to the hydroxylapatite includes DNA–DNA and DNA–RNA duplexes and a small amount of nonspecifically bound radioactivity. Recover the adsorbed fraction by high salt elution (0.3 M phosphate buffer); dilute to 0.14 M phosphate buffer–0.4% sodium dodecyl sulfate, and repass the solution through hydroxylapatite (60°, 0.14 M phosphate buffer–0.4% sodium dodecyl sulfate). Again recover the adsorbed fraction with high salt elution. (3) This adsorbed fraction contains DNA–DNA duplexes and DNA–RNA hybrids. To recover only the DNA involved in the DNA–RNA hybrids remove any sodium dodecyl sulfate present and dilute to 0.14 M phosphate buffer and treat with RNase (50–100 μg/ml at 57° for 10 hr). Alternatively, this fraction can be diluted to 0.01 M phosphate buffer and treated with the same amount of RNase at 37° for 4–6 hr. The RNase treatment renders the DNA formerly involved in RNA–DNA hybrids incapable of binding to hydroxylapatite under the conditions used. Adjust the mixture to 0.14 M phosphate buffer–0.4% sodium dodecyl sulfate and pass it through hydroxylapatite and recover the DNA fraction which does *not* adsorb. After treating with Pronase, this material was deproteinized with phenol and then concentrated on hydroxylapatite (Kohne, 1968). The product consists of DNA sequences which are expressed as RNA.

Results

The technique utilized here was designed to fractionate DNA on the basis of its differential expression in particular tissues or stages of development (Kohne, 1968). It has been utilized by several investigators to measure the extent of expression of the nonrepeated DNA fraction of several higher organisms (Gelderman *et al.*, 1971; Hahn and Laird, 1971; Brown and Church, 1971; Grouse *et al.*, 1972).

Table I gives values obtained for the extent of expression of the nonrepeated DNA fraction of several different cow tissues. The values in Table I should be taken as only approximate values for the extent of expression of DNA in these tissues. The observed value (3%) for the extent of expression of cow brain DNA is significantly lower than that obtained by Hahn and Laird (1971) for mouse brain DNA (10–12%). They found that the mouse brain must be removed and frozen immediately after death (within several minutes) or a much lower extent of expression was observed. This suggests that a very labile RNA population exists in the brain. The cow brains used for the experiments reported here were not removed until 20–30 min after the death of the cow.

There are several limitations on the type of analysis used in these experiments. From one viewpoint the values could be overestimates. If the DNA sequences involved in RNA–DNA hybrids were only partially covered by RNA the values seen in Table I would be reduced. While it seems unlikely that this

TABLE I: Expression of Nonrepeated Cow DNA.[a]

Tissue Source of RNA	RNA C_0t	Kidney [^{3}H]DNA C_0t	Nonrepeated DNA in DNA–RNA Hybrids (%)
Fetal liver	16,600	3.36	6.5
Adult liver	24,000	1.5	5.2
Fetal brain	18,000	3.1	2.8
Adult brain	10,000	1.0	4.2
Fetal kidney	11,200	1.0	3.9
No RNA		2.7	0.8

[a] All reactions were done at 66° in 0.48 M phosphate buffer and 10^{-4} M EDTA. C_0t is equal to [(optical density at 260 mμ of nucleic acid solution) × (hours of incubation/2)].

TABLE II: Kinetics of Reaction of Tritiated Nonrepeated E-DNA Expressed in the Liver with Liver RNA.[a]

Incubation (hr)	RNA C_0t	[^{3}H]E-DNA Adsorbed to Hydroxylapatite (%)
0		0.5
2.1	370	11
6.4	1,170	15
22	4,000	29
43	7,900	38
71	13,000	44
140	26,000	51

[a] The [^{3}H]E-DNA used was isolated at a RNA C_0t of 16,000 (Table I). The reaction was done at 66° in 0.48 M phosphate buffer–10^{-4} M EDTA. Little or no DNA–DNA reaction occurred. Slowdown of the reaction was not due to RNA breakdown occurring during the long incubation period. Liver RNA at high concentration (15 mg/ml) was incubated in the absence of [^{3}H]DNA for 200 hr in 0.48 M phosphate buffer–10^{-4} M EDTA at 66°. Tritiated nonrepeated DNA was then added and the mixture incubated further and the kinetics of reassociation monitored. The kinetics indicated that little damage had been done to the RNA during the 200-hr incubation.

is the case, as the DNA species used in these experiments were small (~400 nucleotides), this point has yet to be resolved.

From another view, the values in Table I could be underestimates. RNA–DNA hybrid formation is time dependent and the reaction may have been stopped before it was completed. This possibility was examined for the E-DNA sequences of liver. Table II shows the kinetics of reaction of isolated liver E-DNA sequences re-reacted with liver RNA. The liver E-DNA was originally isolated at an E-DNA C_0t of 16,000. If all possible E-DNA sequences had reacted by that RNA C_0t then on re-reaction to an RNA C_0t of 17,000 the E-DNA sequences should be 100% reacted. Table II shows that this does not occur and that the reaction is only about half over at an RNA C_0t of 26,000. This suggests that for liver the extent of expression (6%) could be at least doubled. No detectable DNA degradation occurred during the manipulations described above.

Rationale of Experimental Approach for Amplification Studies. A basic assumption in this study is that DNA sequences, if amplified, are amplified in order to provide more templates for transcribing RNA. This clearly seems the case for the amplified ribosomal RNA genes in oocytes. The experimental design for this study involves: (a) the isolation of specific nonrepeated sequences which have been expressed (as RNA) in a particular tissue; (b) measurement of the average number of copies per cell of the E-DNA sequences present in the DNAs of different tissues. Suppose, for example, that the nonrepeated DNA sequences expressed as RNA in the liver have been preferentially amplified ten times. These same sequences would not be amplified in other tissues and would be present just one time per haploid cell in tissues other than the liver. The concentration of these E-DNA sequences is then ten times higher in liver DNA than in the DNA from a different tissue. The rate of reassociation of a particular DNA sequence is proportional to the concentration of that sequence in the total DNA. Isolated liver E-DNA sequences, in the example above, will reassociate ten times more rapidly when mixed with liver DNA than when mixed with the DNA of another tissue since the concentration of these sequences is ten times higher in liver DNA than in DNA from other tissues. The same basic rationale and technique were used to (a) determine whether the multiple ribosomal RNA genes of *E. coli* and *Proteus mirabiles* were similar or different (Kohne, 1968) and (b) to detect the number of SV-40 viral genomes present in a transformed cell line (Gelb *et al.*, 1971) and to study the amplification of hemoglobin genes (Bishop *et al.*, 1972).

To determine the extent of amplification, comparisons were made of reassociation rates obtained when E-DNA is allowed to react with two different DNAs: (a) DNA from the same tissue used to obtain the RNA utilized in E-DNA isolation; (b) DNA from a different tissue. ^{14}C-Labeled *total* nonrepeated cow DNA was included as an internal standard in each incubation mixture to gain greater accuracy of comparison. The ratio of nonradioactive to radioactive DNA purposely was made high to ensure that the rate of reassociation was determined by the nonradioactive DNA. The mixtures were denatured and incubated in 0.48 M phosphate buffer at 66°. Samples were taken at various times after the start of the reaction, and the amount of reassociation of ^{3}H- and ^{14}C-labeled DNA with unlabeled DNA was determined by hydroxylapatite fractionation. The individual rate constants for the reaction could then be determined. Reassociation kinetics of nonrepeated DNA closely follows that of a second-order reaction, and the rate constant can be determined from the relationship $c/C_0 = 1/(1 + KC_0t)$, where c/C_0 is the fraction of unreassociated DNA, K is the rate constant, t is the time of incubation in hours, and C_0 is the initial concentration of unreassociated DNA. The K values for ^{3}H and ^{14}C reactions were calculated for each kinetic point (Table III) and the ratio of the rate constant for the [^{3}H]E-DNA (K_H)[3] and the rate constant for the total nonrepeated DNA $K_{^{14}C}$ was used as an indication of differences in the reaction rate of the [^{3}H]- and [^{14}C]DNAs. Table III presents the data for amplification checks on three different tissues, adult and fetal liver and adult brain. The $K_{^3H}/K_{^{14}C}$ ratios for these experimental reactions closely resemble those for the control reaction in all three cases. This indicates that in each case the majority of the expressed DNA sequences could not be detected as being amplified. The detection technique used could easily have detected a twofold amplification of all of the expressed DNA sequences.

TABLE III: Kinetics of Reaction of Isolated Expressed [^{3}H]DNA Sequences with Different DNAs.[a]

	Incubation (hr)	Unlabeled DNA C_0t	Fraction Adsorbed (%) to Hydroxylapatite		$K_{^3H}/K_{^{14}C}$
			[^{3}H]E-DNA	[^{14}C]DNA	
I. Fetal calf liver E-DNA (6% of total nonrepeated DNA) treated with unlabeled calf liver DNA	19	386	40	32	1.36
	44	924	55	46	1.43
	72	1510	61	53	1.44
	140	2940	69	57	1.68
unlabeled calf brain DNA (Control)	26	380	46	37	1.53
	54	788	59	50	1.45
	73	1060	72	61	1.66
	126	1500	74	62	1.80
II. Adult brain E-DNA (4% of total nonrepeated DNA) treated with adult brain DNA	20	340	42	27	1.90
	48	820	56	41	1.83
	98	1670	70	55	1.90
calf thymus DNA (control)	20	300	36	26	1.6
	40	720	52	40	1.6
	98	1470	67	55	1.7
III. Adult liver E-DNA (5% of total nonrepeated DNA) treated with adult liver DNA	6	228	19	21	0.91
	10	380	28	29	0.97
	26	990	44	50	0.79
	100	3800	63	67	0.86
calf thymus DNA (control)	6	192	19	23	0.77
	10	320	26	33	0.72
	26	832	43	48	0.82
	100	3200	67	72	−0.79

[a] Reactions were done at 66° in 0.48 M phosphate buffer–10^{-4} M EDTA. ^{14}C-Labeled total nonrepeated DNA (calf kidney) was added to each incubation mix as an internal standard. C_0t is defined in Table I. Theoretically, if no amplification has occurred, the $K_{^3H}/K_{^{14}C}$ observed should be equal to one. In practice this is difficult to achieve due to technical reasons. The DNAs were sheared separately and a fourfold difference could exist in piece size between the various DNA preparations. The maximum extent of reaction can also differ for the different DNA preparations and varies from 80 to 90%. Both of these factors would contribute to the ratio not being equal to one. The ^{14}C-labeled nonrepeated DNA was added as an internal standard to control such variables.

Evolution of Expressed DNA Sequences. Previous studies on the evolutionary divergence of DNA have utilized either the repeated or nonrepeated fraction of the DNA (Hoyer and Roberts, 1967; Britten and Kohne, 1967; Laird *et al.*, 1969; Kohne *et al.*, 1972). A major difficulty in the interpretation of experiments with these DNA classes is that virtually nothing is known about their function(s). It is not known, for example, how much of the DNA is actually transcribed during the life cycle of any higher organism. It is difficult then to properly interpret any differences seen between the DNAs of different species.

In the studies reported here the isolation of nonrepeated sequences expressed as RNA (E-DNA) in several cow tissues has enabled us to examine the evolutionary divergence of DNA sequences which actually are used by the cell to make RNA. Specifically, we have asked whether the DNA sequences which can be detected as being expressed (E-DNA) are more or less conserved than the bulk of the DNA sequences in the cell. Nonrepeated DNA sequences held in common between two species should be the direct descendants of a single sequence present in their most recent common ancestor. Thus, differences seen between nonrepeated sequences of different species are due to mutational events occurring since the divergence of these species. Complementary DNA strands from different animal species can interact to form a "hybrid" reassociated DNA–DNA complex. The experiment is designed so that one strand of this "hybrid" molecule is radioactive and from one species, while the other is nonradioactive and from a different species. If the two strands are only partially complementary in the reassociated region, the "hybrid" will have a lower thermal stability than perfectly base-pair matched DNA. The difference in thermal stability between the "homologous" and "hybrid" DNA–DNA duplexes is a measure of the extent of nucleotide sequence changes which have occurred between the two species since their divergence.

To determine the extent of divergence of the E-DNA relative to that of total nonrepeated DNA, small quantities of [^{3}H]E-DNA from a specific cow tissue and *total* ^{14}C-labeled

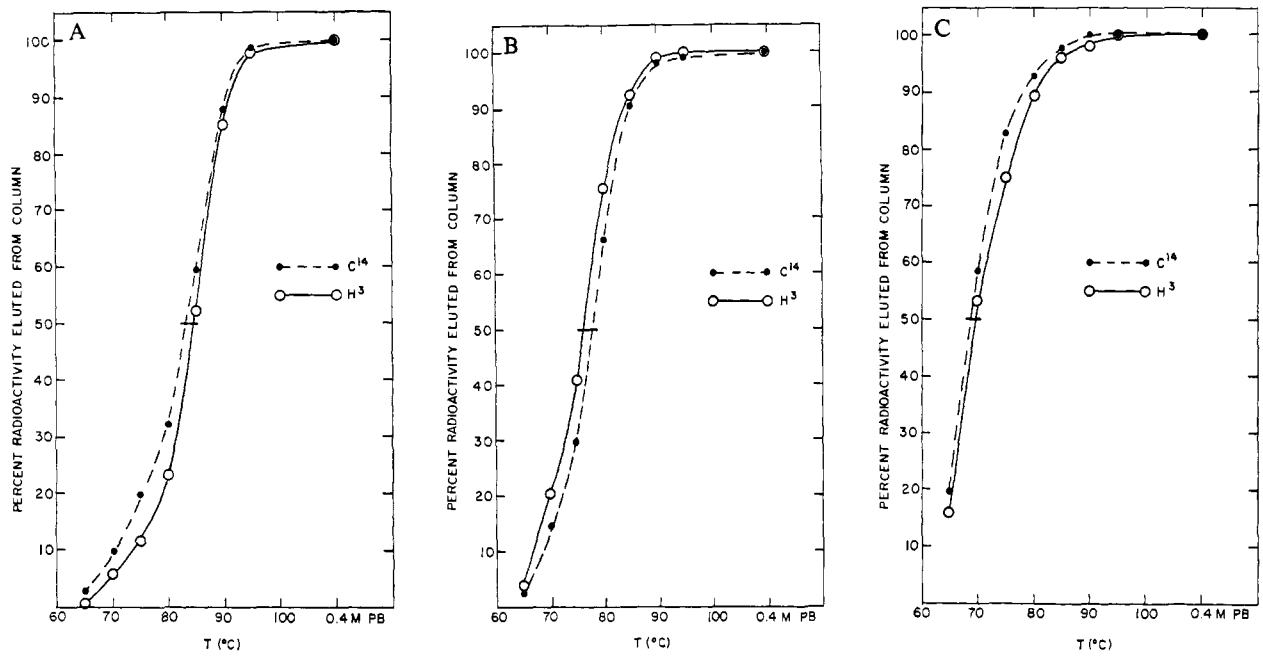

FIGURE 1: Thermal stability profiles. (A) Thermal stability profiles of adult brain [^{3}H]E-DNA (this fraction is about 3% of the total nonrepeated DNA) and ^{14}C-labeled-total nonrepeated DNA treated with unlabeled calf thymus DNA. The DNAs were mixed together and denatured at 100° for 1–2 min. The solution was then made to 0.48 M phosphate buffer and incubated at 66° to an unlabeled DNA C_0t of 2340. The unlabeled DNA to [^{3}H]- and [^{14}C]DNA ratios were both greater than 3000:1. The mixture was then diluted to 0.14 M phosphate buffer–0.2% sodium dodecyl sulfate and passed over a hydroxylapatite column equilibrated to 0.14 M phosphate buffer–0.2% sodium dodecyl sulfate, 60°. Under these conditions single-stranded DNA washes through the column while double-stranded DNA adsorbs. The thermal stability of the adsorbed material was measured by increasing the temperature of the column in 5° steps (Kohne, 1968; Miyazawa and Thomas, 1965); after each temperature increase the column was washed with 0.14 M phosphate buffer–0.2% sodium dodecyl sulfate in order to remove any DNA which had become single-stranded due to the temperature increase. Sixty-two per cent of the [^{14}C]DNA and 71% of the [^{3}H]DNA adsorbed to the hydroxylapatite. The difference in the extents of binding of the [^{3}H]- and [^{14}C]DNA is probably due to a difference in the DNA piece size of the two preparations. The lower thermal stability of the [^{3}H]DNA in each case is very likely a result of radiation damage to the DNA. The [^{3}H]DNA had a very high specific activity while the [^{14}C]DNA specific activity was low: (●) [^{14}C]DNA; (○) [^{3}H]DNA. (B) Thermal stability profiles of cow adult brain [^{3}H]E-DNA and ^{14}C-labeled total nonrepeated DNA treated with unlabeled sheep DNA. This experiment was performed as described in A. Fifty-two per cent of the [^{14}C]DNA and 66% of the [^{3}H]DNA adsorbed to the hydroxylapatite. The unlabeled sheep DNA C_0t was 1800: (●) [^{14}C]DNA; (○) [^{3}H]DNA. (C) Thermal stability profiles of cow adult brain [^{3}H]E-DNA and ^{14}C-labeled total nonrepeated DNA treated with unlabeled pig DNA. The experiment was performed as described in A. Nineteen per cent of the [^{14}C]DNA and 30% of the [^{3}H]DNA adsorbed to the hydroxylapatite. The unlabeled DNA C_0t given was 1800: (○) [^{3}H]DNA; (●) [^{14}C]-DNA.

nonrepeated cow DNA were mixed with large quantities of unlabeled sheep, pig, and cow DNAs, respectively. The mixtures then were incubated until most of the nonlabeled DNA reassociated. The radioactive DNAs in these mixtures are at such low concentrations that these sequences can reassociate only if there are sequences complementary to them in the nonradioactive DNA. At the end of the reassociation period the double-stranded DNA was isolated on hydroxylapatite. Virtually all radioactive DNA in the reassociated fraction is in the form of hybrid DNA molecules. Comparison of control thermal stability profiles with those of E-DNA sequences allowed to react with sheep and pig DNA indicates the extent of sequence divergence. If the same number of changes have occurred in the E-DNA sequences as have occurred in the total DNA sequences the thermal stabilities of the E-DNA and total DNA "hybrid" duplexes will be the same.

Figure 1 gives the thermal stability profiles for the reaction of adult brain [^{3}H]E-DNA and ^{14}C-labeled total nonrepeated cow DNA sequences allowed to react with either cow, sheep, or pig DNA. The thermal stability of the interspecific "hybrid" ^{3}H or ^{14}C duplexes is much lower than that of the control or homologous DNA–DNA duplexes. This indicates that a large amount of sequence divergence has occurred since the divergence 25 million years ago of cow from sheep, and 55 million years ago of cow from pig (Laird *et al.*, 1969). The thermal stability profiles of both the [^{3}H]- and [^{14}C]DNA sequences allowed to react with cow unlabeled DNA are very similar. A similar situation exists for the [^{3}H]- and [^{14}C]DNA allowed to react with unlabeled sheep and pig DNAs where in each case the ^{3}H and ^{14}C thermal stabilities are quite similar (Figure 1). A comparable situation is seen when fetal calf liver E-DNA is allowed to react with pig and sheep DNAs (results not shown). About 6% of the fetal liver nonrepeated DNA is detected as being expressed. These results indicate that expressed DNA sequences have diverged to essentially the same extent as the bulk of the nonrepeated DNA.

Discussion

This report presents the initial experiments in an attempt to determine whether "gene amplification" plays a role in development. The first goal was to determine whether amplification generally occurred for all the DNA sequences transcribed as RNA in various tissues. While not absolutely excluding a small amount of amplification the data show that an amplification similar to that occurring for the ribosomal RNA genes of *Xenopus oocytes* does not occur for the cow E-RNA sequences studied. Gene amplification may be reserved for those cases where a great amount of specific gene product is needed, such as during amphibian oogenesis. However, it is possible that an amplification mechanism does exist for certain genes during development. A source of difficulty in the

interpretation of these experiments is that the function of the RNA transcribed from the nonrepeated E-DNA sequences is not known. It is probable that the majority of the E-DNA is homologous to nuclear RNA and that the cytoplasmic RNA is poorly represented. Perhaps some RNA transcribed into protein arises from amplified sequences. This difficulty could be avoided by using purified mRNA to isolate E-DNA. Alternatively, the fractionated RNA could be radiolabeled and the experiments done by examining the kinetics of the labeled RNA with DNA from different tissues (Gelderman *et al.*, 1971). Bishop *et al.* (1972) have used this technique to examine the amplification of hemoglobin genes. Another approach, using reverse transcriptase to copy purified hemoglobin mRNA, has been tried by Harrison *et al.* (1972) and Packman *et al.* (personal communication). Both of these approaches indicate that there is no large scale amplification of hemoglobin genes. Neither, however, can rule out a twofold amplification of hemoglobin DNA in these cells.

It appears, then, that the nucleotide sequences which can be detected as being expressed have not been significantly more conserved over evolutionary time than the bulk of the nonrepeated sequences. The significance of this observation unfortunately is not clear since the function of the E-DNA is unknown. It may be, for example, that only those DNA sequences directly involved in specifying a protein are conserved to any extent.

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