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Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure

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ABSTRACT

A dot hybridization method is presented for rapidly determining the relative concentrations of nucleic acids in a mixture, as well as the extent of sequence homology between related RNA or DNA species.

INTRODUCTION

Standard hybridization or reassociation procedures are the methods of choice for exactly calculating nucleic acid concentrations, or cross-reactions resulting from sequence homology. However, only semi-quantitative estimates for a large number of samples are often necessary. In this case, it is convenient to use a dot hybridization technique[1,2,3] as described here. Multiple samples of cloned DNAs, identical in amount, are spotted next to each other on a single nitrocellulose filter, in dots of uniform diameter. The filter is then hybridized with a radioactive probe, such as an RNA or DNA mixture which may contain the corresponding sequences in unknown proportions. Conditions are chosen to avoid saturation of the filter-bound DNA. The extent of hybridization with each of the DNA dots is evaluated semi-quantitatively after autoradiography, by visual comparison to a standard consisting of a dilution series of radioactive DNA, similarly spotted on a nitrocellulose filter in dots of the same diameter. This approach is widely applicable to studies on changing populations of RNA during development and, more generally, for convenient evaluation of the relative abundance of nucleic acid sequences in a mixture. Moreover, dot hybridization under progressively more stringent conditions can be used for semi-quantitative

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evaluation of the extent of similarity between homologous sequences. This approach is widely applicable to studies of evolution at the nucleic acid level.

MATERIALS AND METHODS

Preparation of Filters

Nitrocellulose filters (22 mm diameter) are water washed and placed on a platform consisting, from bottom to top, of dry paper towels, moist 3 MM paper and moist nitrocellulose paper. The filters are washed with drops of 1 M ammonium acetate, sucked through by capillarity because of the paper towels. The assembly is covered to prevent drying of the filters.

Plasmid DNA is linearized by restriction endonuclease treatment and digested with Proteinase K (200 µg/ml in 50 mM Tris-HCl, pH 7.5; at 37°C, 30 min each with 0.2% and 2% SDS). After phenol extraction the DNA is denatured in NaOH (usually 0.3 to 0.4 N) for 10 min and chilled, and when needed is diluted with an equal volume of cold 2 M ammonium acetate to a concentration of 16 µg/ml. Samples of 50 µl are taken up in a disposable 100 µl pipet (Clay Adams Micropet), which is then connected through air-filled polyethylene tubing to a water-filled syringe attached to a syringe pump (Model 355; Sage Instruments). With the DNA solution withdrawn several mm above the pipet tip, the pipet is held vertically just above the filter. The syringe pump is turned on, and the pipet is firmly pressed against the filter just as the sample reaches the tip. Although we prefer this procedure for its reproducibility, it is also possible to deliver the DNA manually, using a pipet connected to a Clay Adams pipet filler, directly or through tubing. After each sample is spotted (approximately 1.5 min), the filter is rinsed through with a drop of 1 M ammonium acetate. At the end the filters are washed with approximately 200 ml 4xSSC, to remove specks of dust which, if dried onto the filter, may generate background problems. The washed filters are air dried, shaken in 2X Denhardt's solution[4] for at least 1 hr, drained and air-dried again, baked under vacuum at 80°C for 2 hr, and stored dry.

Hybridization

The filters are soaked for at least 1 hr in 10X Denhardt's solution, 4X SET(1X SET buffer is 0.15 M NaCl, 0.03 M Tris-HCl, pH 8.0, 1 mM EDTA), transferred to a sterile siliconized scintillation vial containing blank hybridization mixture (50% deionized formamide, 2X Denhardt's solution, 4X SET, 0.1% SDS, 100 µg/ml yeast tRNA and 125 µg/ml poly(A)), incubated for at least 1 hr at the hybridization temperature, and then incubated for 16 to 48 hr with gentle shaking in hybridization mixture (approximately 0.5 ml per filter), containing in addition the radioactive probe. A washed blank filter is included at the top of the experimental filters, to minimize their contact with air, and the scintillation vial is capped tightly to prevent evaporation during hybridization.

Washing and Melting

The filters are washed at the incubation temperature with at least two changes of blank hybridization mixture and then thoroughly with 50% formamide-2X SET (final change with shaking at room temperature for 30 min). They are drained, placed on wax paper on top of a glass plate, wrapped with Saran-wrap, and autoradiographed. For melting, the filters are heated for 5 to 15 min in 2.5 to 5 ml melting buffer (50% formamide-2X SET). To minimize irreversible binding of the probe, they are never allowed to dry.

Other

Other materials and methods were as in reference 3.

RESULTS AND DISCUSSION

Reproducibility of DNA Binding

Using this technique, multiple samples of denatured DNA (up to approximately 1 µg/mm^2) can be spotted on a single nitrocellulose filter, in dots of uniform diameter. Binding is essentially quantitative (95-99%) with 1 M ammonium acetate containing 0.02 to 0.2 N NaOH (pH 7.8 to 9.0; Figures 1A, 1B). Ammonium acetate is decidedly better than sodium chloride (pH ca. 12-13) in this respect (Figure 1A).

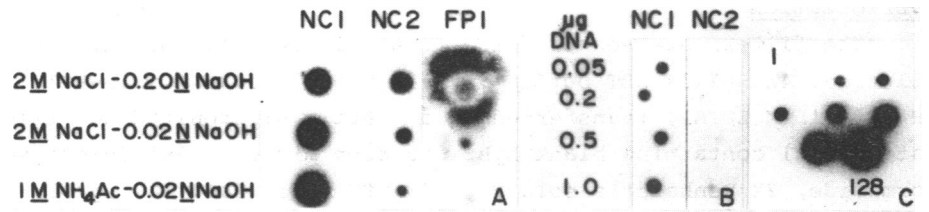

Figure 1. Quantitative formation of DNA dots on filters.
A: Nick translated ^{32}P-labeled DNA was denatured with NaOH and diluted with NaCl or NH_4Ac to the indicated final concentrations. It was then spotted with 100 µl pipets (diameter 1.2mm) on nitrocellulose paper (NC1), stacked on top of a second piece of nitrocellulose paper (NC2), Whatman 3MM filter paper (FP1), and paper towels. Autoradiography revealed that the best binding medium is 1 M NH_4Ac - 0.02 N NaOH (98% binding to NC1), whereas NaCl allows considerably more flow-through, especially at high NaOH concentrations.
B: Labeled DNA was bound as in A but after mixing with the indicated amount of unlabeled, Hind III-digested pML-21 DNA. The binding medium was 1 M NH_4Ac - 0.1 NaOH. Autoradiography revealed that even with the highest DNA load, binding is essentially quantitative.
C: ^{32}P-labeled pML-21 DNA ($2x10^6$ cpm/µg) was bound in 1 M NH_4Ac - 0.2 N NaOH, in a two-fold dilution series. After autoradiography, individual dots were cut and counted, verifying the good accuracy of the dilution series. The lowest amount of DNA spotted was 198 cpm (spot 1, upper left corner). Relative to that, the expected vs. observed radioactivities of the other spots were 2 vs. 2.2 X; 4 vs. 4.7 X; 8 vs. 7.8 X; 16 vs. 15.9 X; 32 vs. 33.6 X; 64 vs. 61.2 X; and 128 vs. 121.1 X (lower right corner).

Figure 1C shows a series of standard dots, made with a dilution series of radioactive DNA. Reproducibility is documented by the excellent agreement of expected and observed radioactivity in the dots (see Figure Legend). Clearly, the relative amount of radioactivity in each dot can be estimated visually with an accuracy of better than two-fold, and over a 100-fold range, taking into account both intensity and apparent diameter of the autoradiographic spots. In our experience, as few as 5 cpm per dot can be detected and evaluated dependably above background.

Measurements of Relative RNA Concentrations by Dot Hybridization

Dilution series of the type shown in Figure 1C can be used as standards, for evaluating the extent of hybridization between radioactive RNA probes in solution and cold DNA dots bound to filter.

It can be easily calculated that, when the filter-bound DNA is in sequence excess, the reaction is pseudo-first order, and the extent of hybridization, H (amount of RNA probe hybridized at time t) should be

$$H=R_o(1-e^{-kD_o t})$$

where R_o and D_o are initial single-stranded concentrations of RNA probe and filter-bound DNA, respectively, and k the hybridization rate constant (inversely related to complexity). Thus, for DNAs of comparable complexities, at any time the cpm hybridized by the various dots should be proportional to the respective R_o, (i.e., to the initial concentrations of the corresponding RNA sequences in the hybridization mixture). This theoretical expectation was confirmed by a reconstitution experiment, using cRNAs prepared from pML-21 and RSF1030 plasmids (Table 1).

We routinely use conditions (i.e., values of $kD_o t$) that result in low fractional hybridization, i.e., low values of H/R_o and H/D_o. As can be seen from the above equation, when fractional hy-

Table 1

Determination of Sequence Abundance by Dot Hybridization*

% of Sequences in Probe Mixture		cpm Hybridized to DNA Dots	
RSF1030	pML-21	RSF1030	pML-21
0	100	0 -	639 (100%)
20	80	136 (20%)	514 (80%)
50	50	319 (47%)	326 (51%)
80	20	526 (78%)	122 (19%)
100	0	677 (100%)	0 -

*cRNAs were prepared[3] separately from pML-21 and RSF1030 plasmids, mixed in various proportions, and hybridized for 24 hr at 55°C with replicate filters containing pML-21 and RSF1030 dots. For both pML-21 and RSF1030, the extent of hybridization using pure probe (639 or 677 cpm; 100% hybridization) corresponded to approximately 0.3% of saturation.

bridization is low, H should increase almost linearly with time. Empirically we have found that under our conditions $D_o t$ values as high as 10^{-2} are satisfactory for sequences with a complexity of 600 nucleotides: in a time-course experiment with silkmoth follicular ^{32}P-RNA and DNA from the chorion cDNA clone, pc401, hybridization was still approximately linear with time at 45 hr and 64° C, with $D_o t$ 10^{-2} and $R_o t < 5x10^{-3}$ (conditions comparable to those of Figure 9 in ref. 3). This is in reasonable agreement with expectations based on similar but RNA-excess reactions described in the literature.[5]

The dot hybridization procedure is not very dependable for detecting extremely rare sequences because of occasional background problems. We have not attempted to determine the detection limit, but have obtained reproducible estimates of relative concentration for sequences accounting for less than 10^{-4} of a mixture.

An example of determining sequence concentrations by dot hybridization is shown in Figure 2. A library of ds-cDNA clones has been produced, each representing one of the many related sequences encoding chorion (eggshell) proteins in silkmoths.[3] Some of these clones are classified here as "abundant" or "rare," depending on the relative concentrations of the corresponding mRNA sequences in cytoplasmic poly(A)$^+$ RNA derived from follicles representing pooled stages of choriogenesis. Experiments at low and high cri-

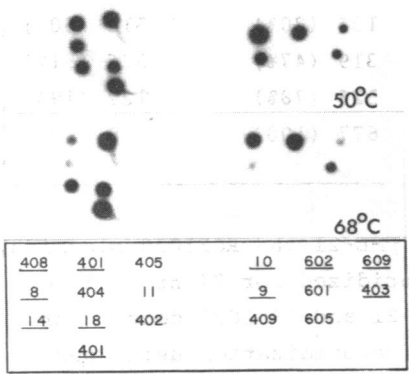

Figure 2. Abundance of various chorion mRNA sequences. Intact end-labeled mRNA from mixed stages of choriogenesis (somewhat deficient in very late stages) was used as probe with filters containing DNA dots from a total of 17 chorion cDNA clones, as shown. After hybridization for 18 hr at 50°C, the filters were washed and autoradiographed, then melted at 68°C and autoradiographed again. Note that only ten clones hybridized at a detectable level under these conditions (underlined); the remaining clones were only detectable with very long exposure. Also note the different abundances scored at the two temperatures (see Text).

terion permitted evaluation of the combined abundance of many related squences, or of the abundance of only specific sequences, respectively.

Clones were dot-hybridized with intact mRNA, end-labeled in vitro with γ-^{32}P-ATP after removal of the 5' terminal cap.[6] After hybridization at 50°C, the filters were washed and melted at progressively higher temperatures and autoradiographed. After the 50°C wash (corresponding to a criterion approximately 20°C below T_m; see below), 10 of the 17 clones scored as abundant, with extents of hybridization varying over an approximately eight-fold range; hybridization with the remaining clones was barely detectable (<1% of maximum). After the 68°C melt, the apparent differences in abundance were accentuated and in some cases reversed: abundance was maximal for pc401 and pc602, and rather high for pc18, pc10, pc14 and pc403 (50% to 10% of maximal), whereas clones pc408, pc609, pc8 and pc9 showed a reduced abundance, only 2-3% of maximal. Whereas at 50°C pc408 scored as a much more abundant component than pc403, at 68°C the opposite was true. Since there are no significant differences in the length and G+C content of various chorion sequences[7] (see also Table 2), the differences in dot intensities at the two temperatures must be due to low-melting mismatched hybrids. Because chorion mRNAs are encoded by homologous multigene families,[3,7,8] the intensity of each hybrid at 50°C scores the combined abundance of mRNAs ranging from identical to rather distantly related to the hybridized clone. By contrast, at 68°C the intensity scores uniquely the abundance of mRNAs that are either identical or very closely similar to the clone sequence. The temperature-dependent differences in intensities with clones pc408 and pc8 vs. clones pc401, pc602 and pc18 have also been verified (data not shown) by quantitative melts (see below).

Discrimination Between Related Sequences

This application of the technique is described in detail elsewhere.[3] Discrimination between related sequences can be accomplished by evaluating either the extent of hybridization or the ΔT_m of dot hybrids. The most convenient procedure is to evaluate, for a series of related DNAs spotted on the same filter, and for a series of replicate experiments at different criteria, the extent

of hybridization (at a time long before saturation) with a limiting amount of a single cRNA probe (see Figure 7 in reference 3). Apparently, mismatching slows down significantly the hybridization reaction, to an extent depending on the criterion, and presumably also on the length of the probe and the degree of clustering of the mismatches. We invariably use filter-bound DNA in excess and operate at $D_o t$ values that are low enough so that the reaction is approximately linear with time even for the DNA dot which is most homologous with the probe. It is also possible to use nick-translated DNA rather than cRNA probes.[2,3]

Melting Profiles of RNA and DNA Hybridized to Dots

Labeled RNA or DNA probes can also be hybridized to filter-bound DNA dots and subsequently washed under progressively more stringent conditions to determine sequence similarities.[1,2,3] The same approach can be used to determine quantitative melting profiles for particular types of hybrids.[2,3] Such profiles help in establishing the most appropriate conditions for dot hybridization. Often one will want to use variable-length probes (such as those generated by fragmentation and end-labeling of RNA, or by cRNA synthesis) without sacrificing the ability to discriminate between related sequences. Variable-length probes ordinarily yield broad melting profiles, whereas discrimination between related sequences is improved when the melting profiles are steep. How can this contradiction be overcome?

Model experiments were performed (Figure 3, Table 2) with dots of hybrid plasmid pβG-1 DNA, which carries as an insertion the nearly complete DNA copy of rabbit β-globin mRNA.[9] The labeled probes used with replicate filters were either β-globin DNA excised from pβG-1 by S1 treatment[10] and end-labeled with γ-^{32}P-ATP; or intact end-labeled β-globin mRNA purified by gel electrophoresis;[6] or alkali-fragmented[3] and subsequently end-labeled β-globin mRNA.

With a long and uniform DNA probe (approximately 580 NT), the melting profile was steep: a temperature increase of approximately 8°C was needed to melt between 10% and 90% of the hybrid (Table 2). The profile was nearly as sharp when intact mRNA (approximately 650 NT) was used as the probe; a slight broadening of

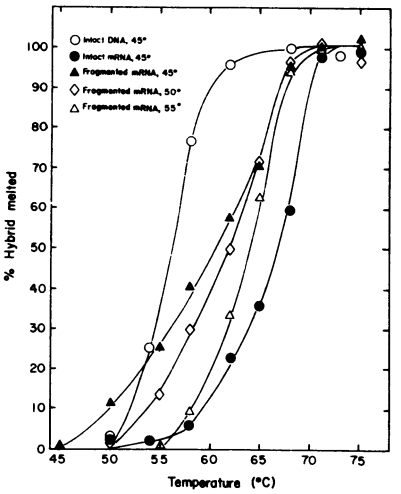

Figure 3. Melting profiles of dot hybrids with intact and fragmented probes. pβG-1 DNA, linearized by treatment with Hind III (which does not cut the β-globin sequence insertion), was bound to filters (0.8 μg/dot). Replicate filters were hybridized for 30 hr at the indicated temperatures with probes consisting either of intact end-labeled β-globin DNA (580 bp, excised from pβG-1), or intact end-labeled globin mRNA (650 NT), or alkali-fragmented end-labeled globin mRNA (approximately 200 NT average length). After the end of hybridization, each filter was washed and melted by stepwise increases in temperature; the filter was counted by Čerenkov counting between melting steps. Note that although the fragmented probe hybrids melt broadly if formed at low criterion, if formed at high criterion they melt as steeply as those formed from intact probes. See also Table 2.

the lower half of the curve might be due to probe fragmentation during some step of the procedure. As expected from the presence of 50% formamide, the RNA-DNA hybrid had a T_m 11°C higher than that of the DNA-DNA duplex.[5]

By contrast, when fragmented RNA was used as probe, the hybrids formed at the same criterion (45°C) melted broadly, undoubtedly as a result of variable duplex length (Figure 3, Table 2). However, for hybrids formed at progressively higher criteria, the corresponding melting curves shifted to higher T_m and became steeper: the melting curve for fragmented RNA hybridized at 55°C was approximately as steep as the curves for intact DNA or RNA, hybridized at 45°C. Presumably, the melting profiles improve as the temperature of hybridization increases because the ability to hybridize is restricted to progressively longer fragments. According to the empirical relationship $\Delta T_m = \frac{650}{L}$ (where L is the length in nucleotides),[11] the effect of variability in duplex length on broadening the melting profile is pronounced only at short average lengths of the hybridized probe.

Melting profiles of chorion sequence hybrids gave similar

Table 2.

Quantitative Melts of Dot Hybrids

DNA on Filter	^{32}P-Probe*	Hybridization Temperature	Corrected Tm**	Steepness of Melt†
pβG-1 (50.9% G+C)	Intact β-globin DNA (580 NT)	45°	59°	8°
	Intact β-globin mRNA (650 NT)	45°	70°	11°
	Fragmented β-globin mRNA (200 NT)	45°	64°	17°
	Fragmented β-globin mRNA (200 NT)	50°	65°	13°
	Fragmented β-globin mRNA (200 NT)	55°	67°	10°
pc408 (56.8% G+C)	cRNA of pc408 insertion (250 NT)	50°	68°	14°
	cRNA of pc408 insertion (250 NT)	55°	68°	13°
pc401 (57.4% G+C)	Intact total chorion mRNA (600 NT)	50°	63°	16°
	Fragmented total chorion mRNA (200 NT)	64°	72°	6°

* The probes used with pβG-1 DNA and pc401 were end-labeled, whereas the cRNA of pc408 was uniformly labeled with α-^{32}P-ATP and α-^{32}P-GTP. Average length of the probes before hybridization are indicated.

** The melting buffer was 0.3 M NaCl and 50% formamide, whereas the hybridization buffer contained 0.6 M NaCl and 50% formamide. To correct for the difference in salt concentration,[12] the experimentally determined T_m has been adjusted by adding 3°C.

† Degrees to melt between 10% and 90% of the hybrid.

results (Table 2; see also Figure 6 in reference 3). pc408 cRNA: pc408 DNA hybrids formed at approximately 13°C below T_m melted with a moderately steep profile, similar to that of fragmented globin mRNA : pβG-1 DNA hybrids formed at approximately 15°C below T_m (Table 2). The importance of hybridization criterion for steepness of the melting curve was most clearly evident in experiments involving pc401 DNA and total chorion mRNA (Table 2). The hybrids formed at low criterion (50°C or approximately 23°C below the calculated T_m of the self-hybrid) were broadly melting, even when intact end-labeled mRNA was used as the probe; similar results were obtained for several additional clones (pc18, pc10, pc408, pc602; data not shown). By contrast, hybrids were very steeply melting if formed at high criterion (approximately 8°C below T_m) between pc401 and fragmented total chorion mRNA. Such steeply melting hybrids must involve sequences which are identical or very nearly homologous to the filter-bound DNA, and which are specifically selected out of the mixed mRNA probe. Thus, even for related sequences such as chorion, the dot hybrids formed with an mRNA mixture should reflect specifically the abundance of each respective sequence in the mixture, provided that the criterion of hybridization or washing is sufficiently stringent (Figure 2 and reference 3).

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