Isolation and Characterization of DNA–DNA and DNA–RNA Hybrid Molecules Formed in Solution†

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ABSTRACT: A simple method for the isolation and characterization of DNA–DNA and DNA–RNA hybrid molecules formed in solution was developed. It was based on the fact that, in appropriate salt concentration, such as 5% Na_2HPO_4, DNA in either double-stranded (DNA–DNA or DNA–RNA) or single-stranded forms, but not free nucleotides, can bind to diethylaminoethylcellulose disc filters (DE81). Thus tested samples were treated with the single-strand-specific nuclease S1 and then applied to DE81 filters. The free nucleotides, resulting from degrading the single-stranded molecules, were removed by intensive washing with 5% Na_2HPO_4, leaving only the hybrid molecules on the filters. The usefulness of this method was illustrated in dissociation and reassociation studies of viral (SV40) or cellular (NIH/3T3) DNAs and DNA–RNA hybrid molecules. Using this technique the reassociation of denatured SV40 DNA was found to be a very rapid process. Dissociation studies revealed that the melting curves of tested DNAs were dependent on salt concentration. Thus the melting temperatures (t_m) obtained for SV40 DNA were 76 °C at 1 × SSC (0.15 M NaCl–0.015 M sodium citrate) and 65 °C at 0.1 × SSC, and for NIH/3T3 DNA 82 °C at 1 × SSC and 68 °C at 0.1 × SSC. MuLV DNA–RNA hybrid molecules were formed by annealing in vitro synthesized MuLV DNA with 70S MuLV RNA at 68 °C. The melting temperature of this hybrid in the annealing solution was 87 °C. Another important feature of this procedure was that, after being selectively bound to the filters, the hybrid molecules could efficiently be recovered by heating the filters for 5 min at 60 °C in 1.5–1.7 M KCl. The recovered molecules were intact hybrids as they were found to be completely resistant to S1 nuclease.

Molecular hybridization as a tool for the detection of specific genes in cells has been widely and efficiently used in the last few years (Winocour, 1971; Spiegelman et al., 1975). This technique is based on the fact that duplexes are formed between various single-stranded DNA molecules or between single-stranded DNA and RNA molecules if sufficient homologous base sequences are present in the interacting molecules (Schildkraut et al., 1961; Hall and Spiegelman, 1961). In most experiments, the DNA is immobilized on nitrocellulose filters and the labeled interacting molecule, whether DNA or RNA, is present in solution (Gillespie and Spiegelman, 1965). In some experiments the entire hybridization mixture is present in solution and the duplexes formed are separated by filtration through nitrocellulose filters (Nygaard and Hall, 1964; Levine et al., 1970). However, small fragments will not attach to the filters (Haas et al., 1972). Another problem is that DNA and hybrid molecules are selectively lost from the filters (Haas et al., 1972).

Other methods which do not use the nitrocellulose filter technique have been recently developed. Hydroxylapatite has been successfully used for the separation of both DNA–DNA (Britten and Kohne, 1968) and DNA–RNA hybrid molecules (Green et al., 1971) formed in solution. It is also possible to degrade single-stranded DNA or RNA with S1 nuclease (Sutton, 1971), while leaving true duplexes intact. In both cases, the radioactivity present in the hybrid is determined by trichloroacetic acid precipitation. However, in our hands, under these conditions, a considerable amount of material is usually lost, and the results are not completely reproducible. In this communication we describe a simple method based on the direct application of hybrid molecules formed in solution to DEAE[1]-cellulose filters. This method is useful even with DNA fragments ranging from 4 to 8 S. Furthermore, the hybrid molecules can be eluted from the filters intact for further analysis.

Experimental Procedure

Preparation of Nucleic Acids. (a) NIH/3T3 DNA. NIH/3T3 mouse fibroblasts were seeded at 1×10^6 cells per 100-mm tissue culture dish (Nunc, Denmrk) in EM + CS. The cultures were kept at 37 °C in a humidified incubator supplemented with a constant flow of 10% CO_2. Two days after seeding, the medium was removed, cells were washed once with PBS, and 1.5 ml of EM + CS supplemented with 10 μCi/ml of [^{3}H]-thymidine (New England Nuclear, specific activity 51 Ci/mM) was added per dish. After 1 h, the dishes were placed on ice, the cultures washed three times with ice cold PBS, and the cells were removed with a rubber policeman. DNA was extracted as described by Collett et al. (1975). The final DNA solution (in 0.1 × SSC) at a concentration of 1 mg/ml was subjected to sonication for 5 min at an output of 40 W (B-12 sonifier, Branson Sonic Power Co.). The size of the fragmented DNA was determined in 5–20% alkaline sucrose gradient made in 0.7 M NaCl–0.3 N NaOH–0.001 M EDTA. The fragments ranged in size from 4 to 7 S.

(b) SV40 DNA. [^{3}H]SV40 DNA (form I) was kindly supplied by Moshe Oren from the Section of Virology, The

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[1] Abbreviations used: EM + CS, Dulbecco modified Eagle's Medium supplemented with 10% calf serum; PBS, phosphate-buffered saline (Salzberg et al., 1973); MuLV, Moloney strain of murine leukemia virus; 1 × SSC, 0.15 M NaCl–0.015 M sodium citrate; t_m, melting temperature; cpm, counts per minute; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; NTE, 0.1 M NaCl–0.01 M Tris-HCl (pH 7.4)–0.001 M EDTA; Tris, tris(hydroxymethyl)aminomethane.

Weizmann Institute of Science, Rehovot, Israel. BSC-1 monkey kidney cells were infected with 50 PFU/cell of plaque purified SV40. At 20 h postinfection, the cells were labeled with 15 μCi/ml of [^{3}H]thymidine (20 Ci/mM; The Radiochemical Center) and harvested at 60 h postinfection. Viral DNA was extracted according to the procedure described by Hirt (1967). The DNA was further purified by CsCl–EtBr gradients and by a neutral sucrose gradient (Laub and Aloni, 1975). The final DNA solution (in 0.1 × SSC) at a concentration of 0.5 μg/ml was subjected to sonication for 7 min at an output of 40 W.

(c) MuLV RNA and [^{3}H]DNA. NIH/3T3 cells releasing MuLV were grown in EM + CS in Roux bottles. Every 12 h the medium was collected, immediately cooled in ice, and centrifuged at 1000g for 10 min and at 40 000g for 2.5 h in a Spinco rotor No. 19 (Beckman). The final pellet was resuspended in NTE buffer (0.1 M NaCl–0.01 M Tris-HCl (pH 7.4)–0.001 M EDTA) and placed on a two-layer cushion made of 1 ml of 65% sucrose in NTE and 1 ml of 15% sucrose in NTE. After centrifugation for 35 min at 105 000g in the Spinco rotor SW39, the virus band which sedimented to the top of the 65% sucrose layer was carefully collected, resuspended in NTE, and rerun on a similar cushion. The virus band was collected, resuspended in NTE, and placed on a linear 15–60% sucrose gradient in NTE. After centrifugation for 60 min at 105 000g, a clear virus band appeared about one-third from the bottom of the tube. The virus was collected, resuspended in NTE, and sedimented to the bottom of the tube as described above. The resulting pellet was resuspended in an appropriate buffer for either the extraction of 70S RNA or the preparation of MuLV [^{3}H]DNA as previously described (Salzberg et al., 1973), with the exception of 0.5 mM $MnCl_2$ replacing 8 mM $MgCl_2$ and 60 μg/ml actinomycin D (Sigma) being added to the endogenous reaction for the preparation of [^{3}H]DNA.

Annealing and Denaturation of Nucleic Acids. SV40 and NIH/3T3 [^{3}H]DNA preparations were first tested for their duplex form. Appropriate samples (750–2000 cpm) were diluted in hybridization mixture (0.6 M NaCl–0.05 M Tris-HCl (pH 7.4)–0.002 M EDTA) in a total volume of 50 μl. S1 nuclease prepared as described by Sutton (1971) was added at a concentration sufficient to completely degrade the same amount of denatured DNA. The entire mixture was diluted in S1 nuclease buffer (0.03 M sodium acetate (pH 4.5)–0.003 M $ZnSO_4$–0.3 M NaCl–100 μg/ml calf thymus DNA–4 μg/ml denatured calf thymus DNA) in a total volume of 100 μl, incubated for 45 min at 37 °C and placed on DE81 disc filters as described in the next paragraph. Resistance of 85–100% was considered an indication of a perfect duplex structure. If, however, a digestion of over 15% of the DNA was observed, the DNA preparation was first incubated in hybridization mixture for 18 h at 67 °C in sealed glass capsules, and a sample containing 1000 cpm was digested with S1 nuclease and applied to DE81 disc filters. Following such treatment, usually over 90% resistance was obtained.

MuLV DNA–RNA hybrid molecules formed in solution were isolated by incubating 600–700 cpm of MuLV [^{3}H]DNA with 0.1 μg of 70S RNA in hybridization mixture (total volume of 50 μl) for 42 h at 68 °C in sealed glass ampules, followed by digestion with S1 nuclease and application to DE81 disc filters. Resistance (70–80%) to S1 nuclease digestion was usually obtained under these conditions.

In order to obtain denatured hybrid molecules, samples of the various tested duplexes were diluted in the hybridization mixture in sealed glass ampules, heated for 15 min at 95 °C, and cooled immediately in ice for 10 min. The samples were then diluted in S1 nuclease buffer (total volume 100 μl), treated with S1 nuclease as described above and applied to DE81 disc filters.

Application of Samples to DE81 Disc Filters. In most cases, 2.5-cm DE81 disc filters (Whatman) were used. One-hundred microliter samples were evenly distributed on each filter with a micropipet. The filters were placed in a beaker and 5 ml per filter of 5% Na_2HPO_4 was added. After 4 min with occasional shaking at room temperature, the solution was carefully decanted, fresh solution added, and the same washing procedure was repeated four times. Two additional washings with the same volume of distilled water were followed. The filters were dried under an infrared lamp and placed in a toluene based scintillation fluid containing 3 g/l. 2,5-diphenyloxazole and 50 mg/l. 1,4-bis[2-(5-phenyloxazolyl)]benzene (Merck), and radioactivity was determined in a Packard TriCarb liquid scintillation counter.

In few cases, 150-μl samples were distributed on 3.5-cm DE81 disc filters, and 8 ml of washing solutions per filter was used.

Elution of Nucleic Acids from DE81 Disc Filters. At the end of the washing procedure described above, the filters were dried and placed in 10-ml glass vials. One milliliter of 0.5 M KCl (in 0.01 M Tris-HCl (pH 7.4)–0.002 M EDTA) was added. After 3 min with occasional shaking, the solution was carefully removed and 1 ml of a higher concentration of KCl (1 M) was added. The same procedure was repeated with gradually increasing concentrations of KCl (Table II). The separate elution fractions were kept in ice, 50 μg/ml of calf thymus DNA was added, total nucleic acids were precipitated with 0.6 M trichloroacetic acid and collected on 2.4-cm 3MM filters (Whatman), and radioactivity was determined in a liquid scintillation counter.

After the final elution step (2.0 M KCl), the filter remaining in the vial was dried and the radioactivity determined as described. In order to compare the radioactivity present on the two different types of filters used (DE81 and 3MM), the same amount of sheared NIH/3T3 [^{3}H]DNA was treated with S1 nuclease and either applied directly to ten individual DE81 disc filters or trichloroacetic acid precipitated and filtered through ten individual 3MM filters. The amount of radioactivity detected on DE81 filters (approximately 2500 cpm) was 50 ± 5.2% higher than that detected on 3MM filters. Similar differences were detected with SV40 [^{3}H]DNA and MuLV [^{3}H]DNA. Hence, normalized values were determined accordingly for the radioactivity present on 3MM filters. It should be emphasized that the difference in the amount of radioactivity detected on both filters is probably not due to differences in the counting efficiency between these types of filters. This fact was established by spotting a known amount of radioactivity ([^{3}H]thymidine) on five individual 3MM or DE81 filters. The filters were dried and *total* radioactivity was determined in a liquid scintillation counter as described above. The same counting efficiency was obtained with both types of filters.

In some experiments, DE81 filters containing hybrid molecules were treated with 1.7 M KCl (in 0.01 M Tris-HCl (pH 7.4)–0.002 M EDTA) at 60 °C. The eluent was dialyzed for 18 h against 1000 volumes of 0.1 × SSC at 4 °C with three changes. The samples were now divided into two groups. One group was kept in ice while the other was heated at 95 °C for 15 min and cooled immediately for 10 min. Both groups were either treated with S1 nuclease as described in the previous section (total volume 2 ml) or diluted in S1 nuclease buffer

TABLE I: Binding of DNA to DE81 Disc Filters.[a]

Source of DNA		Input cpm Bound	cpm Bound after S1 Digestion	% Resistance to S1
NIH/3T3 DNA	Native	1300	1280	98.5
	Denatured	1100	58	5.3
SV40 DNA	Native	600	540	90.0
	Denatured	500	45	9.0
MuLV DNA (single stranded)		800	45	5.6

[a] DNA preparations were diluted in hybridization mixture, and 50-μl samples were diluted in S1 nuclease buffer. The samples were either applied directly to DE81 disc filters (input cpm) or incubated first with S1 nuclease. Specific activities of the DNAs were: NIH/3T3 DNA, 3250 cpm/μg; SV40 DNA, 2×10^5 cpm/μg; MuLV DNA, 2×10^7 cpm/μg.

without addition of exogenous nuclease. The final radioactivity was determined by trichloroacetic acid precipitation.

Results

Binding of DNA to DE81 Disc Filters. It was first essential to establish whether differences in the bindig efficiency to DE81 disc filters exist between native and denatured DNA molecules. Secondly, it was important to demonstrate the specific binding of actual DNA molecules and not merely of single nucleotides. Native and denatured [^{3}H]DNA from three different sources were, therefore, tested for its binding properties to the filters. Each DNA preparation was divided into two samples. One was applied directly to the filters and input radioactivity was determined, while the second was applied to the filters following treatment with S1 nuclease. The results presented in Table I very clearly indicate that being in the native or denatured state does not affect the binding efficiency of cellular (NIH/3T3) or viral (SV40) DNA to DE81 filters since no difference exists in the input cpm bound. However, a dramatic difference exists in the amount of radioactivity bound following treatment with S1 nuclease. When NIH/3T3 or SV40 native DNA is treated with S1 nuclease, between 90 and 98% of the input cpm still binds to the filter, indicating that the majority of the molecules in the sample are complete duplexes. On the other hand, less than 10% of the input cpm bind to the filter when denatured DNA is treated with S1 nuclease. As indicated in Experimental Procedure, MuLV [^{3}H]DNA is prepared in vitro in the presence of actinomycin D. The results presented in Table I demonstrate that at least 95% of the DNA synthesized under these conditions is single stranded and susceptible to S1 nuclease digestion.

Determination of Reassociation Kinetics and Melting Temperatures (t_m) of DNA-DNA and DNA-RNA Hybrid Molecules. The specificity of binding to DE81 disc filters was further tested in experiments in which reassociation kinetics of denatured DNA and the t_m of native DNA or DNA-RNA hybrid molecules were determined. In all experiments described in this section, samples were applied directly to the filters following treatment with S1 nuclease.

SV40 DNA has been chosen for reassociation kinetics studies since its structure and physical properties are well documented and it has been extensively used for mapping studies with restriction enzymes (See Tooze, 1973). As shown

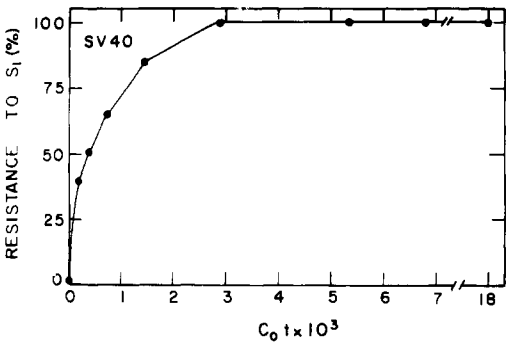

FIGURE 1: Reassociation kinetics of SV40 DNA. Samples containing 750 cpm of SV40 [^{3}H]DNA (see Table I for specific activity) were diluted in the hybridization mixture, heat denatured, and incubated at 68 °C in sealed glass ampules. At various times thereafter, the samples were withdrawn, treated with S1 nuclease, and placed on DE81 disc filters.

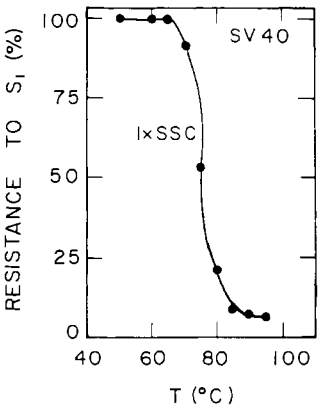

FIGURE 2: Melting curve of SV40 DNA. Samples containing 750 cpm of SV40 [^{3}H]DNA were diluted in 1 × SSC, sealed in glass ampules, heated at the indicated temperatures for 5 min, cooled immediately, treated with S1 nuclease, and applied to DE81 disc filters.

in Figure 1, the amount of radioactivity which is specifically adsorbed to DE81 disc filters increases rapidly with the time of incubation of SV40 denatured DNA. Within 15 min of incubation, already 30% of the input radioactivity became S1 nuclease resistant (C_0t value of 0.2×10^{-3}), and the annealing reaction is completed within 120 min (C_0t value of 2.85×10^{-3}).

In the next set of experiments, the melting temperatures of three different species of DNA-DNA or DNA-RNA hybrid molecules were determined. Samples were heated at the indicated temperature, cooled, digested with S1 nuclease, and applied to DE81 disc filters. The melting curve of SV40 DNA is illustrated in Figure 2. At a salt concentration of 1 × SSC, the t_m of SV40 DNA under our conditions is 76 °C, similar to the t_m established for SV40 DNA-RNA hybrid molecules (Hirai et al., 1971). The t_m of SV40 DNA at 0.1 × SSC under our experimental conditions was about 65 °C (unpublished data).

Since the filter technique has been demonstrated to be highly effective and reproducible in isolating SV40 DNA duplexes, we then determined the t_m of a different and more complicated species of DNA. Purified NIH/3T3 [^{3}H]DNA was treated under identical conditions as above and applied to the filters following digestion with S1 nuclease. A typical melting curve at two different salt concentrations is shown in Figure 3. A t_m of 68 °C at 0.1 × SSC and of 82 °C at 1 × SSC is observed. These values are slightly higher than those established for

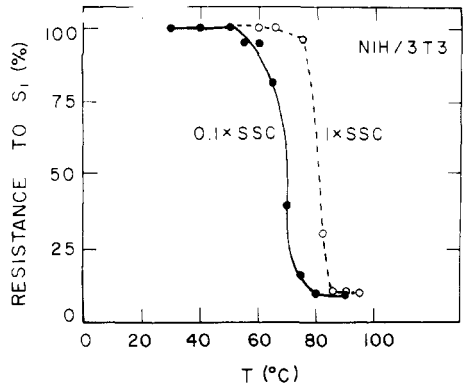

FIGURE 3: Melting curve of NIH/3T3 DNA. Samples containing 2000 cpm of NIH/3T3 [^{3}H]DNA (see Table I for specific activity) were diluted at the indicated salt concentrations and treated as described in Figure 2.

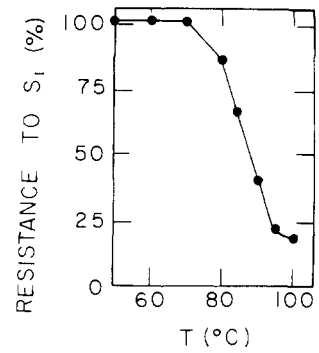

FIGURE 4: Melting curve of MuLV DNA–RNA hybrid molecules. MuLV [^{3}H]DNA (600 cpm) prepared in vitro (see Table I for specific activity) was annealed with MuLV 70S RNA in the hybridization mixture. The mixture was then heated at the indicated temperature and treated as described in Figure 2.

SV40 DNA, indicating a different G-C content in both DNA populations. However, the difference of over 10 °C in the t_m values of NIH/3T3 DNA at 0.1 × SSC and 1.0 × SSC is characteristic for duplex molecules of different types (Aloni, 1972).

Finally, the t_m of MuLV DNA–RNA duplexes was determined. Since these duplexes were formed in vitro, the characterization of the melting curve was particularly important, as it reflects the true nature of the duplex. Figure 4 represents the melting curve obtained at 0.6 M NaCl, the salt concentration used in the hybridization mixture. A typical t_m value of 87 °C was obtained under our experimental conditions.

Thus, with three different types of duplexes, we could demonstrate the specific binding of the duplex to DE81 disc filters following digestion with S1 nuclease.

Elution of Hybrid Molecules from DE81 Disc Filters. One major advantage of the described procedure for the isolation of hybrid molecules would be the possibility of eluting the molecules from the filters in their native form under appropriate salt concentration and temperature. Samples of NIH/3T3 [^{3}H]DNA in hybridization mixture were treated with S1 nuclease, applied to DE81 disc filters, and subjected to different KCl concentrations at different temperatures. The radioactivity eluted from the filters was determined by acid precipitation and normalized values (see Experimental Procedure) were calculated. These normalized values were essential in order to compare the radioactivity eluted from the filter with the radioactivity remaining on the filter. The results shown in Table II indicate that at 60 and 80 °C the majority of radioactivity is eluted at salt concentrations up to 1.7 M KCl. Practically no material is eluted at 0.5 M KCl and, at 2.0 M KCl, only small amounts of radioactivity are still eluted. Similar results were obtained with MuLV RNA–DNA hybrid molecules, with 72% of the radioactivity eluted at 1.5 M KCl at 60 °C.

It was now essential to determine whether the material eluted was still in a hybrid form. Filters containing 2000 cpm of NIH/3T3 [^{3}H]DNA or 700 cpm of MuLV [^{3}H]DNA–RNA duplexes were immersed in 1.7 M KCl at 60 °C, the material eluted was dialyzed, and a sample was treated with S1 nuclease. Another sample was first heat denatured and then similarly treated with S1 nuclease. As shown in Table III, the material eluted is completely resistant to S1 nuclease but becomes sensitive following denaturation.

Discussion

The specific binding of DNA synthesized in vitro in the reverse transcriptase reaction to DEAE-cellulose filters (Salzberg et al., 1973) led us to investigate whether this binding capacity is general for any type of DNA, either single or double stranded. If so, the binding can also be useful for the detection of DNA–DNA and DNA–RNA hybrid molecules formed in solution. We could demonstrate that indeed single- and double-stranded DNAs bind with the same efficiency to DE81 disc filters, even when the DNA is fragmented (4–7 S). The binding appears to be specific to polynucleotides since less than 10% of the input radioactivity remains on the filters, when denatured or single-stranded DNA is digested with S1 nuclease.

The determination of the reassociation kinetics of SV40 DNA and the t_m's of various duplexes by their binding efficiency to the filters also demonstrate the specificity of the binding. For example, the reannealing of SV40 DNA appears to be a very fast reaction, similar to that reported by Sack et al. (1973). Typical melting curves were obtained with three

TABLE II: Elution of NIH/3T3 DNA from DE81 Disc Filters.

Temp of Elution	cpm[c] Eluted at Specified KCl Concn (M)[a]						DE81[b] Filter	Total cpm	% Eluted
	0	0.5	1	1.5	1.7	2.0			
25	9	21	124	206	189	140	1075	1764	39
40	5	23	193	281	182	59	871	1614	46
60	5	27	278	402	356	83	385	1536	75
80	10	24	301	446	262	114	406	1563	74

[a] Filters containing NIH/3T3 [^{3}H]DNA were immersed in 0.01 M Tris (pH 7.4), 0.002 M EDTA, and the indicated concentration of KCl. At each concentration, the filters were immersed for 3 min. Acid-insoluble cpm were determined. [b] At the end of the immersion procedure, the filters were dried and counted. [c] Normalized values (see Experimental Procedure).

TABLE III: Properties of the Material Eluted from DE81 Disc Filters.[a]

Source of Material Eluted		cpm Eluted	cpm Resistant to S1	% Resistance to S1
NIH/3T3 DNA	Before denaturation	850	835	98.2
	After denaturation	810	53	6.5
MuLV DNA-RNA duplexes	Before denaturation	360	325	90.3
	After denaturation	320	30	9.4

[a] Filters were immersed in 1.7 M KCl for 5 min at 60 °C. The eluent was dialyzed and divided into two portions. One was kept in ice and the other heat denatured. Both were diluted in S1 nuclease buffer and each divided again into two samples. One was incubated with no further treatment (cpm eluted), while the other was incubated with S1 nuclease. Trichloroacetic acid precipitable cpm were determined.

different types of duplexes. The t_m of 76 °C at 1 × SSC established for SV40 DNA is similar to that established for SV40 RNA-RNA duplexes isolated from SV40 infected BSC-1 cells late after infection (Aloni, 1972).

The filter technique has also been used to characterize the melting profile of MuLV DNA-RNA duplexes. The determination of the t_m of such duplex molecules is important since MuLV DNA is synthesized in vitro and its viral specificity can be determined only by molecular hybridization to the RNA used as a template for its synthesis (Spiegelman et al., 1975). The melting curve obtained (Figure 4) and the t_m value of 87 °C demonstrate the stability of the hybrid molecule formed in solution (Spiegelman et al., 1975). Thus, it appears that the filter method described is useful for the isolation of duplexes of various types. Moreover, these duplexes can be eluted in their native form from the filters, denatured, and further analyzed. We could demonstrate that no traces of S1 nuclease are present in the eluent since the amount of acid-insoluble radioactivity eluted remained constant following denaturation and incubation with no exogenous S1 nuclease added.

DE81 disc filters can be obtained in various sizes and, thus, applied for different volumes of reaction mixture.

We conclude that the technique described in this communication is highly efficient, simple, and reproducible and can serve as a useful tool for the isolation and characterization of hybrid molecules formed in solution.

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