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Titration of Integrated Simian Virus 40 DNA Sequences, Using Highly Radioactive, Single-Stranded DNA Probes†

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Nick-translated simian virus 40 (SV40) [^{32}P]DNA fragments (>2 × 10^8 cpm/μg) were resolved into early- and late-strand nucleic acid sequences by hybridization with asymmetric SV40 complementary RNA. Both single-stranded DNA fractions contained <0.5% self-complementary sequences; both included [^{32}P]-DNA sequences that derived from all regions of the SV40 genome. In contrast to asymmetric SV40 complementary RNA, both single-stranded [^{32}P]DNAs annealed to viral [^{3}H]DNA at a rate characteristic of SV40 DNA reassociation. Kinetics of reassociation between the single-stranded [^{32}P]DNAs indicated that the two fractions contain >90% of the total nucleotide sequences comprising the SV40 genome. These preparations were used as hybridization probes to detect small amounts of viral DNA integrated into the chromosomes of Chinese hamster cells transformed by SV40. Under the conditions used for hybridization titrations in solution (i.e., 10- to 50-fold excess of radioactive probe), as little as 1 pg of integrated SV40 DNA sequence was assayed quantitatively. Among the transformed cells analyzed, three clones contained approximately one viral genome equivalent of SV40 DNA per diploid cell DNA complement; three other clones contained between 1.2 and 1.6 viral genome equivalents of SV40 DNA; and one clone contained somewhat more than two viral genome equivalents of SV40 DNA. Preliminary restriction endonuclease maps of the integrated SV40 DNAs indicated that four clones contained viral DNA sequences located at a single, clone-specific chromosomal site. In three clones, the SV40 DNA sequences were located at two distinct chromosomal sites.

Radioactive simian virus 40 (SV40) DNAs have been used as nucleic acid probes to detect single copies of viral DNA integrated into the genomes of SV40-transformed cells (10, 12). SV40 probes previously used to obtain such measurements generally were duplex DNAs of low specific radioactivity. Thus, large amounts of transformed cell DNAs (drivers) were required to obtain quantitative information when hybridization assays were performed in solution. Nick-translated [^{32}P]- or [^{3}H]DNAs have substantially increased the sensitivity of duplex DNA probes (16). When nick-translated SV40 [^{32}P]-DNAs were used as probes, viral DNA present as single-copy sequences within as little as 100 μg of SV40-transformed mouse cell DNA could be quantitated by kinetic measurements (23). However, the kinetic approach using duplex DNA probes requires that properties of the probes be extremely reproducible to detect the small rate differences expected for single-copy DNA sequences. For example, disparity between the single-strand lengths of drivers and tracers are known to affect the kinetics of hybridization reactions (5) and can compromise assumptions implicit in the analysis of such reactions.

As an alternative to duplex probes, single-stranded probes offer unique advantages for quantitative hybridization analysis. Because they lack self-complementary sequences, only single-stranded nucleic acid probes can be added to hybridization reactions in large excess over nonradioactive complementary sequences. Under such conditions, single-stranded probes saturate, and hence titrate directly, complementary nucleic acid sequences. In early attempts at hybridization in probe excess, asymmetric SV40 complementary RNA (cRNA) was used as a probe to titrate viral DNA sequences within transformed cell DNAs immobilized on nitrocellulose filters (30). Those experiments overestimated the abundance of SV40 DNA sequences in the immobilized driver DNAs, because cRNA:DNA hybrids were lost selectively from the filters (11). Single-stranded SV40 and adenovirus DNA probes, radiolabeled in vivo, also were employed to detect viral DNA sequences inte-

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grated into DNA from transformed cells (15, 21). Because the single-stranded probes used in those studies were of low specific radioactivity, the multiple hybridization assays required for kinetic analysis again necessitated large cellular DNA samples.

To measure single copies of viral DNA sequences integrated into small amounts of DNA from SV40-transformed cells, we have combined the methods of nick translation (23), SV40 DNA strand separation (26), and quantitative hybridization analysis using an excess of single-stranded SV40 [^{32}P]DNA probes. In this report we describe the details of single-stranded, nick-translated SV40 [^{32}P]DNA probe preparation and the molecular and reassociation kinetic properties of the final viral [^{32}P]DNAs obtained. We have employed the SV40 [^{32}P]DNA probes to characterize quantitatively the viral DNA sequences integrated into the chromosomes of a series of Chinese hamster lung (CHL) cells transformed by *tsA*, *tsA*-revertant, and wild-type SV40 (18, 19, 28).

MATERIALS AND METHODS

Cells and viruses. Wild-type SV40 strain 776 and SV40-transformed Chinese hamster clones CHLWT15, CHLRA30L1, CHLRA30L2, CHLA30L1, CHLA30L2, CHLA209SL1, CHLA209L5, and CHLA239L1 (18, 19, 28) were obtained from R. G. Martin of the National Institutes of Health. Chinese hamster cells were grown in monolayer cultures at 33°C in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum (GIBCO Laboratories) in a humidified atmosphere of 10% CO_2. The CV-1 VII line of African green monkey cells, obtained from J. H. Wilson of the Baylor College of Medicine, was used as a host for SV40 infections. CV-1 VII cells were grown in Eagle minimal essential medium supplemented with 10% fetal calf serum.

Nucleic acids. Intracellular SV40 DNA I was isolated from cultures of virus-infected CV-1 cells (multiplicity of infection = 0.001 to 0.01). At 12 days after infection, low-molecular-weight DNAs were isolated from infected cell lysates (13), and the DNAs were purified by extraction with phenol saturated with 10 mM Tris-hydrochloride (pH 9.5)–0.1 M sodium acetate. Covalently closed, superhelical viral DNA I was purified further by three rounds of isopycnic centrifugation in cesium chloride-ethidium bromide gradients (22) and velocity sedimentation through 5 to 20% sucrose gradients in TNE buffer (10 mM Tris-hydrochloride [pH 7.4]–0.1 M NaCl–1 mM Na_2EDTA). SV40 DNA I (21S) was recovered from gradients, dialyzed against TNE buffer, and stored as aliquots at 4°C over a drop of $CHCl_3$.

High-molecular-weight chromosomal DNAs were prepared from SV40-transformed Chinese hamster lung cell cultures as previously described (24), with some modifications. Cells were harvested, washed in phosphate-buffered isotonic saline (PBS), and suspended at 10^6 to 2×10^6 per ml in 0.15 M NaCl–10 mM Tris-hydrochloride (pH 9.5)–10 mM Na_2EDTA. The cell suspensions were adjusted to 0.1% Sarkosyl (ICN Pharmaceuticals) and then incubated at 55°C for 30 min. Lysates were treated with pancreatic RNase and autodigested pronase and then extracted with $CHCl_3$-isoamyl alcohol (24:1), as described previously (17). Purified DNAs were dialyzed three times against 100 volumes of TNE buffer for a total of 48 h. Agarose gel electropherograms of these DNAs indicated that they ranged in size from 50 to 100 kilobase pairs (kbp). DNA concentrations in purified preparations were determined spectrophotometrically, assuming that 1 absorbance unit at 260 nm (corrected for light scattering) equals 50 μg of duplex DNA.

To determine the amount of DNA per transformed cell, monolayer cultures were grown to confluence, under which condition all of the cells should have a uniform G_1 complement of DNA (20). Cultures were harvested by treatment with trypsin and EDTA and dispersed to single cells by trituration; cells were counted in a cell counter, model ZBI (Coulter Electronics, Inc.). Cells were dispensed into a series of duplicate tubes, each pair of tubes containing a specified number of cells (0.5×10^6 to 2×10^6), and the DNA content of each tube was determined by the diphenylamine reaction (4).

Asymmetric SV40 cRNA. SV40 cRNA was synthesized at 37°C in a 100-μl reaction mixture that contained 0.25 μg of SV40 DNA I, 0.125 μg of *Escherichia coli* RNA polymerase holoenzyme (Miles Laboratories, Inc.), 0.15 M KCl, 40 mM Tris-hydrochloride (pH 7.9), 6 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 10 μg of bovine serum albumin per ml, and 0.1 mM each ATP, CTP, GTP, and UTP (29). To synthesize [^{32}P]cRNA to high specific radioactivity (1×10^8 to 2×10^8 cpm/μg), the nonradioactive UTP was replaced with 0.007 mM [α-^{32}P]UTP (400 Ci/mmol; New England Nuclear Corp.). Large amounts (0.5 to 1.0 mg) of asymmetric SV40 [^{3}H]cRNA were prepared by the procedure of Sambrook et al. (26). [^{3}H]UTP (6 μCi/mmol; ICN Pharmaceuticals) was included in these reactions to enable us to monitor transcription and recovery of cRNA. Net incorporation of radioactive UTP ceased within 2 to 3 h. Reactions were terminated by digesting the SV40 DNA template with DNase I (Worthington Diagnostics) and cRNA's were recovered (29). SV40 early (E)-strand cRNA sequences, which comprised 95% of the products of these reactions, were resolved from small amounts (5%) of contaminating late (L)-strand RNA sequences by incubating the reaction products to a R_0t value of 20 mol s × liter^{-1} (R_0t is the concentration of RNA [moles of nucleotide liter^{-1}] × time [seconds]) in 0.4 M phosphate buffer (PB) (PB equals equimolar mono- and dibasic sodium phosphate [pH 6.8] measured in a 1 M stock solution)–0.1% sodium dodecyl sulfate (SDS) at 68°C, and then absorbing RNA duplex molecules on hydroxylapatite (Bio-Rad Laboratories). Approximately 90% of the cRNA was eluted from hydroxylapatite as single-stranded cRNA (60°C, 0.14 M PB). SV40 cRNA's were stored as aliquots at −20°C in 0.14 M PB–0.1% SDS–1 mM Na_2EDTA.

Nick translation. SV40 DNA I (10 μg/ml) was treated with DNase I (2.5 ng/ml, 37°C, 2 min) in mixtures that contained 50 mM sodium phosphate

(pH 7.4), 5 mM $MgCl_2$, 10 μM deoxynucleoside triphosphates ([α-^{32}P]deoxynucleoside triphosphates, 200 Ci/mmol; Amersham Corp.), and 100 μg of bovine serum albumin per ml. *E. coli* DNA polymerase I (Boehringer Mannheim Biochemicals) then was added to a final concentration of 115 μg/ml, and the reaction mixtures were incubated at 15°C until approximately 25% of the radioisotope had been incorporated into acid-insoluble nucleic acid. Reactions were terminated by adding Na_2EDTA (100 mM) and heating to 68°C for 10 min. DNAs were extracted with $CHCl_3$-isoamyl alcohol (24:1), and the products were separated from unincorporated substrates by chromatography on Sephadex G-50 (Pharmacia Fine Chemicals, Inc.). [^{32}P]DNAs were recovered by adding 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol. After more than 2 h of incubation at −20°C, DNA precipitates were collected by centrifugation and then dried in a Speed Vac (Savant Instruments, Inc.). Nick-translated [^{32}P]DNAs were redissolved in TNE buffer and stored as aliquots at −20°C. When nick-translated SV40 DNA preparations (2×10^8 to 4×10^8 cpm/μg) were denatured, the resulting single-stranded DNA fragments ranged in length from 350 to 500 nucleotides (M. A. Marchionni, unpublished data).

Strand separation. Asymmetric SV40 cRNA was used to separate the E- and L-strand sequences of nick-translated SV40 [^{32}P]DNA. According to previous convention (15), we define E-DNA as those sequences complementary to SV40 cRNA and to SV40 early mRNA. Our scheme for separating SV40 DNA strand sequences is a modification of the protocol developed by Sambrook et al. (26). Nick-translated [^{32}P]DNA was denatured and mixed with a 20-fold excess of asymmetric SV40 [^{3}H]cRNA. Hybridization was performed at 68°C in 0.14 M PB–0.1% SDS–1 mM Na_2EDTA until 50% of the [^{32}P]DNA hybridized (EC_0t value of 0.4 mol-s × liter^{-1}; EC_0t equals the concentration of DNA [moles of nucleotides liter^{-1}] × time [seconds] corrected to standard conditions [68°C, 0.18 M Na^+]). Hybridization products were fractionated on hydroxylapatite columns, as described previously (1, 3, 10). L-[^{32}P]DNA and single-stranded SV40 [^{3}H]-cRNA eluted at 0.14 M PB; E-[^{32}P]DNA:[^{3}H]cRNA duplex hybrids eluted at 0.4 M PB. Eluates were adjusted to 0.6 N NaOH and incubated at 37°C for 3 h to hydrolyze [^{3}H]cRNA. Samples were neutralized with HCl, dialyzed against TNE buffer for 20 h, and precipitated with ethanol. DNA preparations were redissolved in PSE buffer (50 mM PB, 0.1% SDS, 1 mM Na_2EDTA) and then adjusted to a final Na^+ concentration of 1 M. DNAs were allowed to self-anneal at 68°C to an EC_0t value of 1 to 2 mol-s × liter^{-1} (i.e., 500 to 1,000 times the SV40 $C_0t_{\frac{1}{2}}$; $C_0t_{\frac{1}{2}}$ equals the concentration of DNA [moles of nucleotides liter^{-1}] × time [seconds] under which condition 50% of the single-stranded DNA has formed a duplex) and duplex molecules were removed from the preparation by a second round of hydroxylapatite chromatography. Single-stranded [^{32}P]DNA probes again were recovered from the 0.14 M PB eluates as described above. During the preparation of the single-stranded probes, >90% of the radioactive DNAs were recovered from each step of the procedure. The final yield of single-stranded [^{32}P]DNAs was approximately 50%.

Hybridization assays. High-molecular-weight DNA purified from transformed cells or SV40 DNA I was fragmented for hybridization reactions by sonication in TNE buffer at 0°C. We used a Biosonik IV sonicator equipped with a microtip tuned to 60% of maximum power for 10 min, and obtained DNA fragments of 350 to 450 nucleotides single-stranded length (data not shown). Sonicated DNA samples then were passed through a column of Chelex-100 resin (Bio-Rad Laboratories), and DNA was recovered quantitatively by washing the resin with 3 volumes of TNE buffer. DNAs were collected by ethanol precipitation; after they had been dried and dissolved in deionized, glass-distilled water, DNA concentrations were determined spectrophotometrically. Aliquots were dried in 1.5-ml siliconized polypropylene tubes and either stored at −20°C or used immediately. For hybridization, DNA samples were dissolved in 5 μl of PSE buffer containing 20 μg of salmon DNA and overlaid with mineral oil for heat denaturation (100°C, 10 min). Either E-[^{32}P]DNA or L-[^{32}P]DNA probe (500 pg) was added, and the mixtures were adjusted to 1 M [Na^+] with 3 M NaCl in a final volume of 10.1 μl. Immediately after these additions were made (performed in <60 s), the reactions were brought to 68°C and were incubated for 48 h. Hybridization reactions were terminated by quickly freezing samples in cold methanol (−35°C). Duplex DNA formation was assayed by hydroxylapatite chromatography on 1-ml columns operated at 60°C, as described previously (1, 3, 10).

Digestion of DNAs with restriction endonucleases. All restriction endonucleases were purchased from Miles Laboratories, Inc., except *Taq*I, which was obtained from Worthington Diagnostics. Digestions were carried out under the conditions recommended by the suppliers; enzyme activities were calibrated before use (17). One unit of enzyme is defined as the amount that digests 1 μg of λ DNA completely in 1 h. To obtain complete digestions of transformed cell DNAs, DNAs were incubated with restriction endonuclease (1.0 U/μg of DNA) for 20 h. Digestion products were extracted with $CHCl_3$-isoamyl alcohol (24:1), collected by ethanol precipitation, and dissolved in 10 mM Tris-hydrochloride (pH 7.4)–1 mM Na_2EDTA. One half of each sample was retained for electrophoretic analysis, and the remainder was again digested as described above. Fragments produced by the digestions were analyzed for their contents of SV40 DNA sequences by electrophoresis and the nitrocellulose transfer-hybridization procedure described below. When both digests produced the same set of DNA fragments detected by an SV40 nucleic acid probe, we surmised that both samples had been digested to completion.

Electrophoresis, transfer to nitrocellulose, and nucleic acid hybridization. Samples that contained 10 to 20 μg of transformed cell DNA digestion products were electrophoresed in 1% agarose gels (17) and were blotted onto nitrocellulose filters (BA85, Schleicher and Scheull) for hybridization with SV40-specific nucleic acid probes (27). Filters analyzed with [^{32}P]cRNA probes were processed as described by Ketner and Kelly (14). For hybridization with [^{32}P]DNA probes, filters were preincubated at 68°C without probe in sealed plastic bags containing 6× SSC (SSC: 0.15 M

NaCl–0.015 M sodium citrate), 5× Denhardt solution (8), 0.1% SDS, and 250 to 300 μg of denatured salmon DNA per ml. After 12 to 16 h the fluid within the bags was replaced with fresh hybridization fluid, and denatured [^{32}P]DNA probe (5×10^5 cpm/ml) was added. Plastic bags were resealed and immersed in a shaking water bath at 68°C for 24 h. Filters were washed for 6 h at 68°C in three changes of 2× SSC–0.5% SDS and then washed for 30 min at 45 to 50°C in 0.1× SSC. Autoradiographic exposures were from 1 to 14 days at −96°C with XR-1 film (Eastman Kodak Co.) and Cronex Lightning-Plus intensifier screens (Du Pont Co.).

RESULTS

To study particular nucleic acid sequences present in one copy per mammalian cell (e.g., integrated viral DNAs) requires very sensitive and specific methods. We compared asymmetric SV40 [^{32}P]- or [^{3}H]cRNA and strand-separated SV40 [^{32}P]DNAs as quantitative probes for picogram quantities of SV40 nucleic acid sequences. A substantial difference between RNA and DNA probes derives from the kinetics of RNA:DNA hybridization as compared with DNA:DNA reannealing. Our data, as well as those from other laboratories (9), suggest that RNA:DNA hybridization reactions are slower than DNA:DNA reassociation reactions by an amount dependent on the ratio of RNA to DNA. The hybridization of denatured SV40 DNA with a 10-fold excess of asymmetric SV40 [^{3}H]cRNA appears to be approximately 5-fold slower than the reassociation of SV40 [^{3}H]DNA (see Fig. 4, below). Because detection of single-copy sequences in mammalian DNAs requires extended hybridization incubations (C_0t values of 10^4 to 10^5 mol-s × liter^{-1}), the slow rate of cRNA:DNA hybridizations necessitated prolonged incubation times (10 to 20 days) at 68°C. Under those conditions cRNA probes are unstable. Thus, we decided to separate the E- and L-strand sequences of nick-translated SV40 [^{32}P]DNA and to use them as single-stranded probes for SV40 nucleic acid sequences.

Properties of SV40 cRNA. SV40 cRNA transcribed by *E. coli* RNA polymerase in vitro is highly asymmetric, being complementary to viral E-strand DNA sequences (29). Thus, cRNA has been employed as a hybridization reagent to separate the strands of full-length linear molecules of SV40 DNA (26). Such strand-separated SV40 DNAs radiolabeled in vivo have been used as low-specific-activity hybridization probes to assay SV40 sequences within relatively large samples of transformed cell DNA (15). Strand separation of full-length DNA probes requires that cRNA's contain an extensive, but not necessarily complete, representation of SV40 E-DNA sequences. On the other hand, resolution of highly radioactive nick-translated SV40 DNA fragments (350 to 500 nucleotides) into E- and L-strand sequences requires that cRNA's be derived from all regions of the SV40 genome and that nick translation radiolabels all SV40 DNA sequences. The following several experiments indicate that, by using the methods described, these conditions are satisfied.

A preliminary experiment indicated that approximately 10% of the cRNA was capable of forming RNA:RNA hybrids. After a self-annealing incubation (R_0t value of 20 mol-s × liter^{-1}), complementary RNA sequences were removed as duplex RNAs by hydroxylapatite chromatography. We again assessed the asymmetry of the [^{3}H]cRNA and observed that the final preparations were approximately 99.5% asymmetric (Table 1). To analyze the content of SV40 nucleotide sequences within asymmetric [^{32}P]-cRNA, we digested SV40 DNA with endo R·*Hpa*I + *Eco*RI, and the resulting DNA fragments were electrophoresed in an agarose gel and transferred to nitrocellulose filters. Filters were incubated with [^{32}P]cRNA probe, and the cRNA:DNA hybrids formed on the filters were detected by autoradiography (Fig. 1). We concluded from experiments such as this that asymmetric SV40 [^{32}P]cRNA contains sequences derived from all portions of the viral genome. However, when the autoradiography was within the linear range of the film sensitivity (Fig. 1, lane b), we observed that some SV40 cRNA sequences were more prevalent than others. [^{32}P]cRNA sequences transcribed from the early region of the viral genome (Fig. 1, DNA fragments A and C) were present in greater concentrations than were those from the late region (Fig. 1, fragments B and D).

Separation of the strands of SV40 [^{32}P]-DNA. Nick-translated SV40 [^{32}P]DNA was denatured and mixed with a 20-fold excess of asymmetric SV40 [^{3}H]cRNA to absorb the E-[^{32}P]DNA sequences. The cRNA:DNA hybridization reaction followed pseudo-first-order kinetics and terminated when about 50% of the [^{32}P]DNA had hybridized with the [^{3}H]cRNA (data not shown). Single-stranded nucleic acids,

TABLE 1. *Lack of complementarity of nucleic acid sequences within asymmetric SV40 [^{3}H]cRNA*[a]

Hydroxylapatite column eluate	cpm	% of total
Single-stranded [^{3}H]cRNA (0.14 M PB)	9,404	99.50
Duplex [^{3}H]cRNA (0.4 M PB)	47	0.50

[a] An aliquot of asymmetric SV40 [^{3}H]cRNA was incubated under annealing conditions (0.4 M PB–0.1% SDS, 68°C) to a R_0t value of 20 mol-s × liter^{-1}. Duplex formation was measured by hydroxylapatite chromatography as described in the text.

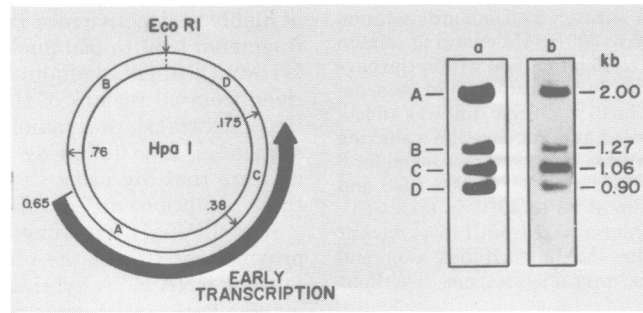

FIG. 1. *Nucleotide sequence composition of asymmetric SV40 [^{32}P]cRNA. Recognition sites for restriction endonucleases HpaI (↑) and EcoRI (↓), as well as for the DNA fragments that result from cleavage by the two enzymes together, are indicated (7). SV40 DNA I was digested to completion with endo R·HpaI + EcoRI. Aliquots that contained 10^{-3} (lane a) and 10^{-4} (lane b) μg of the reaction products were electrophoresed in a 1% agarose gel at 2 V/cm for 10 h. DNA fragments were transferred from the gel to nitrocellulose filters (27). Analysis employing asymmetric SV40 [^{32}P]cRNA probe (10^{8} cpm/μg) was performed as described (14). Autoradiographic exposure shown was for 3 days.*

L-[^{32}P]DNA and [^{3}H]cRNA, and duplex E-[^{32}P]DNA:[^{3}H]cRNA hybrids were resolved by chromatography on hydroxylapatite columns. [^{3}H]cRNA in both fractions was eliminated by alkaline hydrolysis. Most of the self-complementary [^{32}P]DNA sequences within the two fractions of probe were removed by a self-annealing reaction, followed by a second chromatography on hydroxylapatite. Greater than 95% of the L-[^{32}P]DNA sequences and >70% of the E-[^{32}P]-DNA sequences were recovered as single-stranded nucleic acids from these columns.

Nucleotide sequence composition of single-stranded SV40 [^{32}P]DNA probes. To establish that E- and L-strand [^{32}P]DNA sequences were resolved from each other, we carried out the experiment summarized in Fig. 2. In this experiment we compared the reassociation of each probe incubated alone (self-association) and the reassociation of the two probes mixed in equimolar amounts. The self-association indicated that both probes contained <0.5% self-complementary sequences. In general, preparations of L-strand [^{32}P]DNA contained slightly fewer residual self-complementary sequences (<0.2%) than did preparations of E-strand [^{32}P]DNA (<0.5%). When equimolar amounts of the probes were incubated together, the [^{32}P]-DNAs reannealed as a single component displaying second-order kinetics and a $C_0t_{\frac{1}{2}} = 1.9 \times 10^{-3}$ mol-s × liter^{-1}. Thus, the complementary sequences of nick-translated SV40 [^{32}P]DNA were resolved into two single-stranded probes.

Autoradiograms illustrated in Fig. 3 compare the [^{32}P]DNA sequences within E-, L-, and duplex DNA probes and thus identify the regions of the SV40 genome included within each probe. Each of the three probes hybridized to DNA

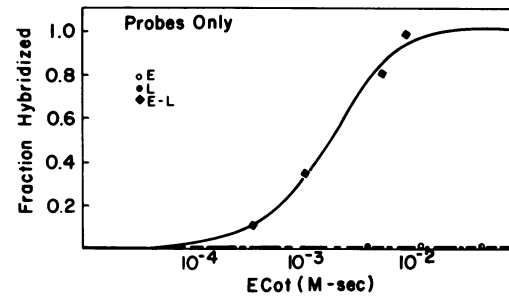

FIG. 2. *Analysis of complementary SV40 [^{32}P]-DNA sequences. Purified single-stranded SV40 E- and L-[^{32}P]DNA probes were incubated under annealing conditions (1 M Na^+, 68°C) either separately or mixed together in equimolar quantities. Symbols are indicated in the figure. At appropriate times after the beginning of the incubation, aliquots were removed, and the fraction of probe that hybridized was determined by hydroxylapatite chromatography as described in the text. Values of C_0t are corrected to standard conditions (3).*

sequences derived from all regions of the SV40 genome. Hence, the nick translation and strand-separation procedures do not result in omission of [^{32}P]DNA sequences. As was observed in the asymmetric [^{32}P]cRNA probe (Fig. 1), DNA sequences from the SV40 early region were preponderant in the duplex and L-strand probes (Fig. 3, fragments A and C). The basis for this sequence selection is not clear at present. Because of the design of our quantitative assay, however, these differences should not affect SV40 sequence titrations (see below).

Single-stranded [^{32}P]DNA probes were next compared with SV40 DNA radiolabeled during infection and with cRNA by an analysis of their

hybridization kinetics (Fig. 4). When we mixed either of the single-stranded [^{32}P]DNA probes with an excess of denatured authentic SV40 [^{3}H]DNA, both probes annealed with the [^{3}H]-DNA at a rate 80 to 90% of the rate of the [^{3}H]DNA reassociation. The small difference in rates did not interfere with detection of picogram quantities of SV40 DNA.

Titration of picogram amounts of SV40 DNA. A large excess (10- to 50-fold) of single-stranded [^{32}P]DNA probes was used to titrate viral sequences within the DNAs of SV40-transformed cells. In these assays, the hybridization reactions were taken to their endpoints, and the amounts of probe rendered duplex were directly proportional to the amounts of SV40 DNA present. A reconstruction experiment demonstrated the usefulness and sensitivity of this assay (Fig. 5). E- and L-[^{32}P]DNA probes both yielded reproducible and comparable titrations of small amounts of SV40 DNA mixed with a large excess of salmon DNA. Both probes were capable of detecting <10 pg of SV40 DNA within samples of 20 μg of total DNA (i.e., 1 part in 2×10^6). Based on linear regression analysis, the ^{32}P-probe rendered duplex in the experiment illustrated was directly proportional to the amount of SV40 DNA in the range of 1 to 100 pg ($r^2 > 0.97$).

Viral DNA sequences integrated in SV40-transformed CHL cell clones. Table 2 summarizes our analyses of eight cloned lines of CHL cells transformed by SV40. Among the

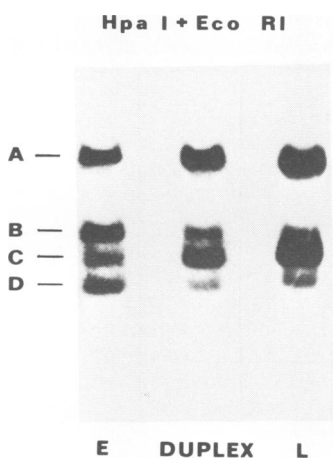

FIG. 3. *Nucleotide sequence composition of SV40 [^{32}P]DNA probes. SV40 (HpaI + EcoRI) DNA fragments (10^{-3} μg) were immobilized on nitrocellulose filters as described in Fig. 1. Conditions of hybridization with [^{32}P]DNA probes (10^8 cpm/μg), as well as the post-hybridization washing procedure, are given in the text. Autoradiography was for 3 days.*

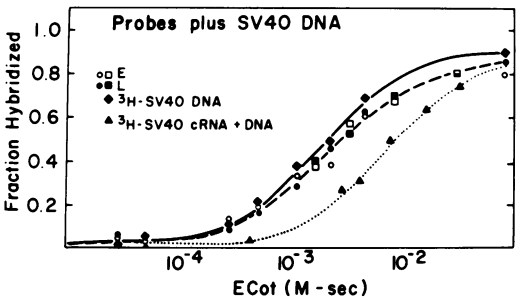

FIG. 4. *Kinetic properties of the single-stranded SV40 [^{32}P]DNA probes and of SV40 [^{3}H]cRNA. SV40 [^{3}H]DNA I (4.8×10^4 cpm/μg; 4.2 μg/ml) was purified from cultures of infected CV-1 cells that had received 50 μCi of [^{3}H]thymidine per ml during the last 2 days of infection (see text). A sample of the [^{3}H]DNA was fragmented (single-strand length, 350 to 400 nucleotides), and then a 1.0-ml aliquot was denatured and split into two portions of 0.5 ml each. Each portion received 5×10^4 cpm of either of two E-[^{32}P]DNA (○, □) or of two L-[^{32}P]DNA (●, ■) probes (10^8 cpm/μg). Mixtures were adjusted to 0.18 M Na^+ and incubated at 60°C. At appropriate times after the beginning of the incubation, the proportions of both [^{3}H]DNA and probe [^{32}P]DNA that had hybridized were determined as described in Fig. 2. For each fraction analyzed, the ^{3}H counts per minute were corrected for spillover (3%) of the ^{32}P counts per minute. Second-order rate constants obtained by regression analysis of these data were used to calculate the theoretical second-order plots indicated by the lines drawn through the data points. Both the [^{3}H]DNA self-reaction (solid line) and the reaction of the single-stranded SV40 [^{32}P]DNA probes with the [^{3}H]DNA (broken line) are displayed. Asymmetric SV40 [^{3}H]cRNA (4×10^7 cpm/μg) was mixed with denatured nonradioactive SV40 DNA at an RNA:DNA ratio of 10:1, and then the hybridization mixture was incubated at 68°C in 0.4 M PB–0.1% SDS. The reaction was monitored by hydroxylapatite chromatography as described above, and the fractions of complementary SV40 DNA sequences hybridized to [^{3}H]cRNA were calculated (▲).*

lines are five clones transformed by *tsA* mutant viruses, two transformed by a *tsA*-revertant virus, and one transformed by wild-type SV40 virus. Six of the transformed cell lines had diploid (or near diploid) genomes, as indicated by both their chromosome numbers and DNA contents. Clone CHLWT15 was approximately tetraploid, and clone CHLA30L2 contained about 1.5 diploid equivalents of DNA per cell.

We used the single-stranded [^{32}P]DNA probes to titrate the SV40 DNA sequences integrated within the chromosomal DNA of these cell lines. Based on the results of those measurements, the clones are heterogeneous with respect to their content of SV40 DNA. Clones containing ap-

proximately 1, 1.5, and 2 viral genome equivalents per diploid CHL genome were recognized (Table 2).

We studied the arrangement of the SV40 DNA sequences integrated within the DNAs of these transformed clones by restriction endonuclease digestion, agarose gel electrophoresis, and Southern transfers as previously described by others (2, 6, 14). In the absence of restriction endonuclease treatment, SV40 DNA sequences in seven of the eight clones (all but CHLWT15) were located only in high-molecular-weight chromosomal DNA (data not shown). Clone CHLWT15 apparently excises SV40-containing DNA fragments, which migrate in electropherograms as a heterogeneous population of molecules in the region of viral DNA I (Fig. 6). The fraction of CHLWT15 cells that liberated free viral molecules was not reduced by repeated subcloning in tissue culture. For this reason, we did not study the integrated viral DNA sequences in this clone further.

Chromosomal DNAs purified from all eight transformed cell clones were digested with restriction endonucleases that do not cleave SV40 DNA I (such as *Bgl*II, *Sma*I, or *Sal*I), and the resulting DNA fragments were analyzed as described above. We expected to observe a single, characteristic DNA fragment containing SV40 homology for each chromosomal site of viral DNA integration (2, 14). In one such analysis, illustrated in Fig. 6, endo R·*Bgl*II (or *Sal* I) was used to digest the DNAs. In autoradiograms, some of which are shown in Fig. 6, we observed that three clones each contained SV40 DNA sequences integrated at a single, clone-specific site in the Chinese hamster genome. The sites of viral DNA integration in each clone can be characterized by sizes of the *Bgl*II (or *Sal*I) fragments that contain SV40 DNA homology: CHLA30L1 (22.5 kbp), CHLA209SL1 (12.6 kbp) and CHLA209L5 (8.25 kbp). For the last-named clone, our data agree with data of others (6). Three other clones contained viral DNA integrated at two apparent sites: CHLRA30L1 (12.8 and 20.1 kbp), CHLRA30L2 (*Sal*I; 11.1 and 30 kbp), and CHLA239L1 (20.0 and 24.0 kbp). Clone CHLWT15 contained SV40 DNA integrated at a single site (20.3 kbp), but as described above, it also contained free viral DNAs. Interestingly, in three independent clones, SV40 DNA sequences were integrated into endo R·*Bgl*II DNA fragments of 20.0 to 20.6 kbp (CHLWT15, CHLRA30L1, and CHLA239L1), and in two clones, the SV40 sequences were integrated within *Bgl*II fragments of 12.6 to 12.8 kbp (CHLRA30L1 and CHLA209SL1). Although these *Bgl*II DNA fragment size similarities originally suggested that multiple, independently transformed clones might have contained SV40 DNA sequences in the same chromosomal site, mapping with other restriction endonucleases indicated that the sites of integration of each of the above clones differ from one another (M. A. Marchionni and D. J. Roufa, submitted for publication).

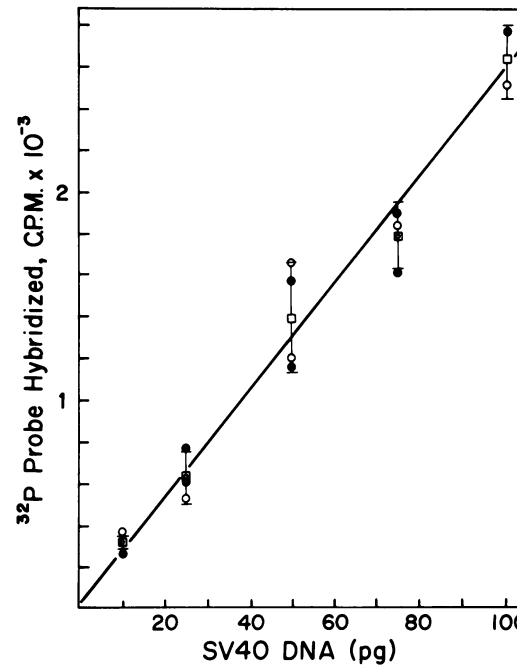

FIG. 5. *Detection of picogram amounts of viral DNA; a reconstruction experiment. Mixtures of various amounts of SV40 DNA (10 to 100 pg) plus 20 μg of salmon DNA (single-strand length, 350 to 400 nucleotides) were denatured and mixed with 500 pg of either of two E-[^{32}P]DNA (○, □) or of two L-[^{32}P]DNA (●, ■) probes. Reannealing reactions were adjusted to 1 M Na^+ and incubated at 68°C for 48 h. In preliminary experiments (not shown) we determined that this incubation was sufficient to obtain at least 95% of the terminal levels of duplex formation between SV40 DNA and probe [^{32}P]DNA. Each sample was analyzed by hydroxylapatite chromatography for the amount of [^{32}P]DNA probe hybridized at completion of the reaction. The values obtained for the probes plus salmon DNA alone (50 cpm for the L-[^{32}P]DNA and 200 cpm for the E-[^{32}P]DNA) were subtracted from all data points.*

DISCUSSION

To study the structure and function of single-copy nucleotide sequences within complex eucaryotic genomes, it is useful to employ a sensitive and quantitative hybridization assay. Interpretations of most solution hybridization assays have been based on the kinetics of tracer molecules that anneal with driver DNAs or RNAs.

TABLE 2. *Analysis of SV40 DNA sequences contained within transformed CHL cell clones*[a]

Cell line	No. of chromosomes[b]	Cellular DNA content[c] (μg/10⁶ cells)	Viral DNA content determined by:			
			Titrations with probe[d]		Endonuclease maps	
			SV40 DNA/CHL DNA ratio (pg/μg)	SV40 genome equivalents per diploid CHL genome[e]	No. of sites[f]	Genome equivalents per site[g]
CHLWT15	33.3 ± 0.82	15.6 ± 1.67	1.49 ± 0.10[h]	2.63 ± 0.18	1[i]	
CHLA30L1	21.0 ± 0.22	10.2 ± 0.42	0.96 ± 0.11	1.60 ± 0.19	1	1.5
CHLA30L2	39.4 ± 0.69	18.9 ± 1.01	0.90 ± 0.05	1.59 ± 0.09		
CHLRA30L1	22.0 ± 0.30		1.23 ± 0.10[h]	2.17 ± 0.18	2	
CHLRA30L2	21.9 ± 0.10		0.55 ± 0.10	0.97 ± 0.18	2	
CHLA209SL1	21.2 ± 0.35	9.95 ± 0.38	0.56 ± 0.16[h]	0.99 ± 0.28	1	1.05
CHLA209L5	21.8 ± 0.18		0.53 ± 0.10[h]	0.93 ± 0.18	1	0.85
CHLA239L1	21.8 ± 0.35		0.74 ± 0.03	1.31 ± 0.05	2	

[a] Data given as the mean ± standard error unless otherwise indicated.

[b] Chromosome numbers for the clones listed in the table were determined in approximately 10 chromosome spreads (25).

[c] Measured in confluent (G_0/G_1) cell cultures by the diphenylamine reaction (4), as described in the text.

[d] SV40 single-stranded [^{32}P]DNA probes were used in 10- to 50-fold excess to titrate viral DNA present in samples containing 10 to 40 μg of DNA from clones listed in the table (Fig. 5).

[e] Based on M_r of SV40 DNA = $3.4 \times 10^6 = 5.64 \times 10^{-6}$ pg and on DNA content of diploid CHL cells.

[f] Equals the number of *BglII* (or *SalI*) DNA fragments that contained SV40 DNA homology.

[g] DNAs from clones CHLA30L1, CHLA209SL1, and CHLA209L5 were digested with several single-site or multiple-site restriction endonucleases or both, and the fragments produced were analyzed by electrophoresis and Southern transfer-hybridization as described in the text. Endonuclease maps were deduced from endo R fragments that contained SV40 DNA homology and from the assumption that the integrated virus is composed of a contiguous, unaltered stretch of SV40 nucleotide sequences (Marchionni and Roufa, submitted for publication).

[h] Determined after growth at both 33°C and 39°C.

[i] Nonintegrated, free viral DNA sequences were contained within most preparations of DNA from this clone.

Consequently, multiple measurements of the fraction of probe rendered duplex versus time generally have been required to calculate the concentration of complementary nucleic acid sequences. In addition, many assays have been performed by using nucleic acid probes with low specific radioactivities such that large amounts of genomic DNA were required for kinetic determinations on single-copy DNA sequences. Those technical constraints, until recently, have precluded a variety of important experiments. For example, DNAs purified from the cells of specific tissues or from cells within particular stages of the cellular life cycle have been difficult to obtain in sufficient quantities for accurate reassociation kinetic measurements. Finally, hybridization studies that rely on kinetic measurements yield approximate estimates of nucleic acid sequence concentrations because they are based on estimations of kinetic rate constants and upon the assumption that all nucleic acid components within a reassociation reaction (driver and probe) behave ideally. Because of these limitations, we were motivated to develop an alternative to the kinetic method for quantitative analysis of single-copy DNA sequences within complex eucaryotic genomes.

We developed an assay to study single-copy viral gene sequences integrated into the DNAs of SV40-transformed rodent cells. We labeled SV40 DNA I with ^{32}P to high specific radioactivity by nick translation (23) and preparatively separated the E- and L-strands of the [^{32}P]DNA on the basis of E-strand–cRNA complementarity (26, 29). The resulting single-stranded SV40 DNA probes both were almost completely free (>99.5%) of complementary DNA sequences (Fig. 2). We found the single-stranded E- and L-strand probes to be preferable to an asymmetric cRNA probe for the several reasons described above. Furthermore, if one resolves viral DNA into specific fragments by restriction endonuclease digestion and gel electrophoresis before nick translation and strand separation, it is possible to obtain nucleic acid probes for discrete regions and strands of the DNA sequence. Such a battery of individual, strand-specific nucleic acid probes is extremely useful for detailed investigations of DNA replication and transcriptional processes within complex eucaryotic genomes (Marchionni and Roufa, submitted for publication).

SV40 cRNA did not contain equimolar amounts of SV40 E-DNA sequence comple-

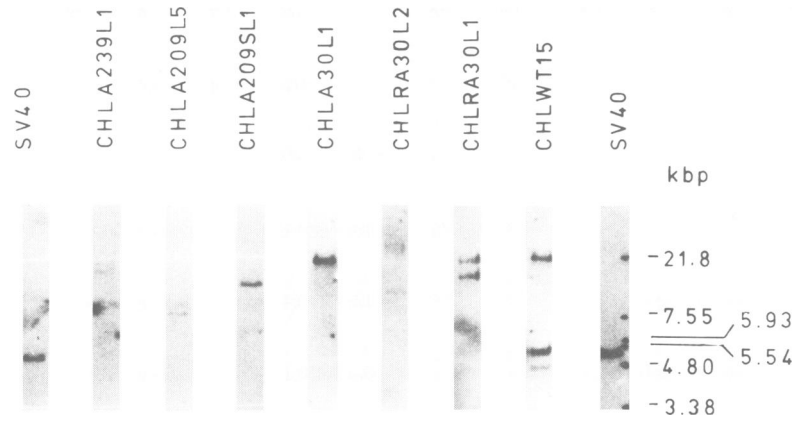

FIG. 6. *Sites of integration of viral DNA within transformed cell chromosomal DNA. DNA (20 μg) purified from SV40-transformed CHL clones was digested to completion with endo R·BglII, with the exception of DNA from CHLRA30L2, which was digested with SalI. Digestion products were electrophoresed in a 1% agarose gel, blotted onto nitrocellulose filters, and immobilized for hybridization with nick-translated SV40 duplex [^{32}P]DNA (2×10^8 cpm/μg). Filters were treated as described in the text. Autoradiography was for 14 days. Positions and lengths of electrophoretic markers of λ DNA digested with EcoRI also are indicated in the figure.*

ments (Fig. 1). Thus, by carrying out [^{32}P]DNA strand separation with a large excess of cRNA, we avoided the omission of specific DNA sequences in the E- and L-strand DNA probes. Although the single-stranded SV40 DNA probes contained nucleic acid sequences derived from all parts of SV40 genome, some sequences clearly were present in greater amounts than were others (Fig. 3). However, a comparison of the reassociation kinetics of E-[^{32}P]DNA with L-[^{32}P]DNA ($C_0t_{\frac{1}{2}}$ value of 1.8×10^{-3} mol-s × liter^{-1}; Fig. 2) and the reassociation of SV40 [^{3}H]DNA purified from infected cells ($C_0t_{\frac{1}{2}}$ value of 2.0×10^{-3} mol-s × liter^{-1}; Fig. 4) indicated that the single-stranded DNA probes contain at least 90% of the DNA sequences within the duplex viral genome.

For detecting small amounts of SV40 DNA sequences (10 to 100 pg) contained within cellular DNA (10 to 100 μg), titrations were carried out in the presence of a large excess of probe (Fig. 5). This insured that all SV40 sequences within the probes were present in sufficient amounts to titrate complementary sequences, although each probe was not an equimolar mixture of all viral sequences (Fig. 3). Despite the large excess of radioactive probe used in these titrations, the stringent conditions of the reactions and of hydroxylapatite chromatography insured low assay backgrounds and high signal-to-noise ratios (Fig. 5). Under these conditions, ^{32}P-probe:SV40 DNA hybrids displayed a melting temperature (T_m) of 86.2°C, which was nearly identical to that of native SV40 DNA duplex (T_m of 86.8°C; data not shown).

We used the single-stranded SV40 [^{32}P]DNA probes to titrate viral DNAs integrated into the genomes of eight, well-characterized, SV40-transformed CHL cell clones (Table 2). These titrations were associated with a relative error of 5 to 30%, a precision significantly greater than has been obtained by kinetic analyses alone (10, 12, 23). Based upon these titrations and upon measurements of the diploid DNA contents of several of the clones, we calculated the number of viral DNA equivalents per diploid cell genome in each clone. CHLWT15 excepted, the clones comprise several groups, based on their contents of integrated SV40 DNA sequence. The first group consists of three clones (CHLRA30L2, CHLA209SL1, and CHLA209L5), each of which contains approximately one genome equivalent of SV40 DNA sequence per diploid cell genome. Based on preliminary restriction endonuclease mapping experiments (Fig. 6; M. A. Marchionni and D. J. Roufa, manuscript in preparation) with CHLRA30L2, the viral DNA sequences are distributed between two chromosomal sites of integration. In the two other clones of this group, SV40 DNA sequences are integrated into a single site each. Three transformed cell clones comprise the second group, and they contain 1.3 to 1.6 viral genome equivalents of SV40 DNA sequence per diploid complement of cellular DNA (CHLA30L1, CHLA30L2, and CHLA239L1). One of these clones (CHLA30L1) contains its viral DNA at a single chromosomal site, and clone CHLA239L1 has its viral DNA distributed between two sites. Clone CHLRA30L1 contains approximately two viral genome equivalents of

SV40 DNA per diploid cellular DNA complement, and these sequences are distributed between two chromosomal sites of integration.

Data summarized in Table 2 do not by themselves impute specific arrangements or compositions to the integrated viral DNA sequences. When considered together with more detailed restriction endonuclease mapping, however, the data provide important quantitative information concerning the viral DNA sequences integrated within the chromosomes of the cells. For example, SV40 viruses genetically identical to the transforming viruses have been rescued from all clones studied with the exception of CHLA209L5 (19). Of the eight clones that we have analyzed by titration with single-stranded [^{32}P]DNA probes (Table 2) and by detailed restriction endonuclease mapping (Marchionni and Roufa, submitted for publication), only CHLA209L5 lacks a complete viral genome. Integrated viral DNA within the chromosomes of this clone lacks most of the genetic sequence encoding the SV40 capsid proteins VP2 and VP3 (0.8 to 0.95 on the SV40 physical map).

The methodology described in this report has allowed us to study molecular details of the replication of SV40 DNA sequences integrated into cellular chromatin and, in particular, to test the effect of functional T-antigen on programming of replication during S phase of the cell cycle (Marchionni and Roufa, submitted for publication). Although these methods were designed specifically around the biochemical properties of SV40 DNA I and asymmetric cRNA (26, 29), the approach we have used should be more broadly applicable. As an example, for cloned genetic sequences derived from purified mRNA's (e.g., cDNA clones), the cDNA's, the purified mRNA's, or asymmetric RNAs transcribed in vitro might be used to strand-separate nick-translated cloned duplex DNAs.

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