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Isolation and Partial Characterization of Single-Stranded Adenoviral DNA Produced During Synthesis of Adenovirus Type 2 DNA

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Single-stranded fragments of adenovirus type 2 DNA were isolated from infected KB cells under conditions which retarded reassociation of complementary sequences but did not denature native viral DNA. Of the total intracellular, virus-specific DNA labeled during a 1-h pulse with tritiated thymidine beginning 15 h after infection, about 20% was single stranded when fractionated on hydroxylapatite. This DNA shifted predominantly to the double-stranded fraction on hydroxylapatite during an extended chase incubation, suggesting that it may represent single-stranded DNA in replicating intermediates. Furthermore, the single-stranded DNA annealed nearly equally to both strands of the adenovirus genome. These findings indicate that at least portions of both complementary strands of adenovirus type 2 DNA are exposed as single strands during the period of viral DNA synthesis.

Mature adenovirus type 2 (Ad2) DNA is a duplex molecule with a molecular weight of about 23×10^6. With one exception describing circular DNA-protein complexes from Ad2 virions and from an avian adenovirus (CELO) (13), the genomes of all adenoviruses appear to be nonpermuted, linear molecules (1, 4, 5, 7, 23), and all adenovirus DNA thus far examined contains an inverted terminal repetition (5, 25).

Several recent reports have focused on the mechanism of replication of the DNA of adenoviruses. Replicating strands of Ad2 DNA isolated from infected HeLa cells were smaller than genome size in alkaline sucrose gradients and, in contrast to Okazaki-type products (10), these strands showed a continuum in size after short pulses indicating sequential addition of single nucleotides to growing chains (8). Replicating DNA of Ad5 synthesized in KB cells or in isolated KB cell nuclei sedimented more rapidly than genome-sized molecules in neutral sucrose gradients and, by electron microscopy, was found to be at least partially single stranded (ss; 20, 22). Some of these molecules appeared as forked structures with varying lengths of ss DNA in one of the arms. In addition, ss viral DNA produced after reversal of hydroxyurea inhibition hybridized preferentially to one of the separated DNA strands (19), and when forked molecules were examined after partial denaturation, strand displacement always began at the adenine-thymine-rich end (18). One model for replication, suggested by Sussenbach et al. (20) to account for these findings, proposed that replication starts from a specific end of a duplex parent molecule and involves the synthesis of one strand with the consequent displacement of its parental counterpart.

Recent experiments in this laboratory, in which the helper effect of Ad2 on the production of the ss DNA of adenovirus-associated virus (14) was investigated, revealed that relatively large amounts of ss Ad2 DNA were produced in cells infected only with Ad2. This Ad2-specific, ss DNA was readily detected by hydroxylapatite chromatography when small numbers of infected cells were sonicated and when DNA was extracted in the cold in the presence of a low concentration of salt, conditions designed to retard reassociation of complementary ss fragments of viral DNA. In the present report we describe the isolation, quantitation, and partial characterization of this ss DNA which is synthesized during Ad2 infection of KB cells.

MATERIALS AND METHODS

Materials. [*Methyl*-^{3}H]thymidine ([^{3}H]dThd, 20 or 40 Ci/mM) and carrier-free ^{32}P were obtained from New England Nuclear Corp., Boston, Mass. Nuclease-free Pronase and dThd were purchased

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from Calbiochem, Los Angeles, Calif; 5-fluorodeoxyuridine (FdUrd) from Hoffman-La Roche, Inc., Nutley, N.J.; Sarkosyl (NL 97) from Geigy Industrial Chemicals, Ardsley, N.Y.; 2'-deoxycytidine-hydrochloride from Sigma Chemicals, St. Louis, Mo.; sodium dodecyl sulfate from BDH Chemicals, Ltd., Poole, England; and Bio Gel HTP hydroxylapatite (HA) from BioRad Laboratories, Richmond, Calif.

Virus and cells. Conditions for the growth of Ad2 in KB cells have been described (2, 16). For preparation of virus labeled with [^{3}H]dThd in DNA, FdUrd was added to a final concentration of 10^{-6} M at 10.5 h after infection, followed by 2.5 μCi of [^{3}H]dThd per ml. For preparation of ^{32}P-labeled viral DNA, phosphate-free Eagle spinner medium supplemented with 5% dialyzed horse serum was used, and 2 μCi of ^{32}P per ml was added 5 h after infection. Virus was harvested after 48 h at 36.5 C and purified as described (16). DNA was isolated from virus and purified as described previously (14, 15).

Isolation of viral DNA from infected cells. KB cells in suspension at 3×10^5/ml were infected with Ad2 at multiplicities between 10 and 100 mean tissue culture infective doses per cell. In most experiments 60 ml of cells in medium without serum were treated with 10^{-5} M FdUrd for 30 min at 14.5 h after infection, then pulsed with [^{3}H]dThd in the continued presence of FdUrd (3). Concentrations of isotopes and variations in the pulse and chase conditions are given below. All subsequent procedures were done in the cold. To keep the concentration of viral DNA low, no more than 3×10^6 cells, in 10 ml, were harvested by centrifugation and lysed in 10 ml of cold, dilute buffer containing 0.6% sodium dodecyl sulfate, 0.01 M NaCl, and 0.01 M Tris-hydrochloride, pH 8.0. The lysate was sonicated, extracted twice with equal volumes of redistilled phenol, and exhaustively dialyzed against 0.01 M NaCl, 0.01 M Tris-hydrochloride, pH 8.0.

HA chromatography. Separation of ss DNA from double-stranded (ds) DNA was performed by HA chromatography essentially as described by Gelb et al. (6). ^{3}H-labeled DNA was diluted 10-fold in 0.14 M phosphate buffer (equimolar mono- and dibasic sodium phosphate, pH 6.8) containing 0.4% (wt/vol) sodium dodecyl sulfate and applied to the HA column. At this buffer concentration, DNA molecules having ds regions are retained, whereas molecules which are entirely ss pass through the column. ds DNA was quantitatively recovered by elution with 0.4 M phosphate buffer. Recoveries of ss and ds DNA were measured by acid precipitation of aliquots, and the fractions were concentrated for DNA-DNA hybridization by vacuum dialysis against SSC (0.015 M NaCl, 0.0015 M sodium citrate).

DNA-DNA hybridization. Hybridizations were carried out on nitrocellulose filters (Schleicher and Schuell, type B6) by the method of Warnaar and Cohen (24). Filters contained 8 to 10 μg of Ad2 DNA. Each reaction vial contained one filter with DNA and one blank filter. All determinations were done in duplicate. Blank fiters retained less than 0.5% of input counts/min.

Separation of Ad2 heavy (H) and light (L) DNA strands. H and L strands of Ad2 DNA were prepared using poly(U, G) as described by Patch et al. (11).

Sucrose gradient sedimentation. DNA was sedimented through preformed, linear 10 to 30% (wt/wt) alkaline sucrose gradients in the SW41 rotor at 193,000 $\times$ g (average) for 17 h at 10 C. Gradients contained 0.7 M NaCl, 0.3 M NaOH, 0.01 M EDTA, and 0.15% (wt/vol) Sarkosyl.

RESULTS

Isolation of intracellular ss DNA from Ad2-infected KB cells. ^{3}H-labeled DNA was isolated from Ad2-infected KB cells and separated into ss and ds fractions by HA chromatography. The distribution of DNA between ss and ds fractions both for total DNA and virus-specific DNA after hybridization is summarized in Table 1. After a 1-h pulse (experiment 1, 2, 3) with or without FdUrd, 15 to 20% of total ^{3}H-labeled counts/min were recovered from HA in the ss fraction. Hybridization of ss and ds DNA fractions to Ad2 DNA showed that 70 to 80% of ss DNA was viral and of the total viral counts per minute about 20% were ss. In control experiments mature Ad2 DNA (^{3}H labeled) was added to a pellet of infected cells at the time of lysis and DNA was extracted in the same manner. Since 99% of the marker DNA fractionated as ds on HA, it appears very unlikely that ss fragments of DNA were artifactually generated from native viral molecules by the low salt buffers, sonication, or HA procedures.

To demonstrate further that the ss ^{3}H-labeled DNA did not represent ds fragments which had failed to adsorb to HA, two additional tests were performed. First, a sample of the ^{3}H-labeled ss fraction was recycled on a second column of HA. More than 90% of the DNA again fractionated as ss. Secondly, a sample of the ^{3}H-labeled ss DNA was reacted in solution with an excess (10 μg) of unlabeled, denatured Ad2 DNA under annealing conditions. After incubation at 68 C in 1 M NaCl for up to 40 h, 70% of the DNA fractionated on HA as ds.

To demonstrate the accuracy of recovery of ss DNA and to show that the extraction procedure did not lead to self-annealing of ss DNA, heat-denatured marker Ad2 DNA (5 μg) was added to infected cells immediately before lysis and extraction. About 80% of this DNA consistently fractionated as ss. Therefore, only a relatively small fraction of ss DNA fragments reassociated during the extraction and fractionation procedures (data not shown).

Efficiency of recovery of viral DNA. The efficiency of recovery of total intracellular viral DNA by the described extraction procedure was determined. Since it was shown previously (12)

TABLE 1. *Hydroxylapatite fractionation of ss and ds fragments of intracellular Ad2 DNA*[a]

Expt	Conditions of pulse[b]	Hydroxylapatite fractionation[c]			Hybridization to ad2 DNA[d]				% Total Ad2-specific counts/min[e]	
		Total counts/min on HA	% counts/min recovered as:		ss Fraction		ds Fraction			
			ss	ds	Input counts/min	% Bound	Input counts/min	% Bound	ss	ds
1	1-h Pulse	569,000	17	83	28,000	70	8,720	50	23	77
	1-h Pulse without FdUrd	387,000	15	85	15,000	75	52,000	54	19	81
2	1-h Pulse	6,110,000	16	84	82,870	67	64,760	51	20	80
	1-h Pulse, 4-h chase	6,000,000	13	87	57,410	72	77,420	53	17	83
	1-h Pulse, 12-h chase	5,900,000	6	94	29,373	60	74,454	61	6	94
3	1-h Pulse	2,780,000	20	80	10,831	82	27,182	67	24	76
	30-s Pulse	20,100	8	91	359	40	3,630	57	6	94
Native DNA control expt[f]		35,990	<1	99						
		7,674	<1	99						
		10,491	<1	99						

[a] A suspension of 3×10^5 KB cells/ml was infected with 10 to 100 mean tissue culture infective doses of Ad2 virus/cell. At 14.5 h after infection cells were treated with FdUrd (10^{-5} M), except where noted otherwise. After pulse labeling as indicated, 3×10^6 cells were pelleted at 4 C from 10 ml and sheared DNA was isolated and extracted with phenol as described.

[b] Cells were pulse labeled with [^{3}H]dThd at 15 h after infection for 1 h using 8.3 μCi/ml (experiment 1) and 20 μCi/ml (experiment 2, 3), and for 30 s using 100 μCi/ml (experiment 3). Labeling and chase conditions for experiment 2 are described in the legend to Fig. 2. In experiment 3, the 30-s pulse was ended by diluting cells into 100 ml of cold (0 C) medium containing 100 μg of unlabeled dThd per ml and immediately pelleting and extracting cells as above.

[c] A 5-ml sample of extracted DNA (ca. 2 optical density units at 260 nm total) was diluted to 50 ml in 0.14 M NaP and 0.4% sodium dodecyl sulfate and chromatographed on HA. Recoveries of ss and ds DNA fractions were quantitated by acid precipitation of a 5-ml aliquot. In all cases recoveries of ^{3}H-labeled DNA were greater than 90% of total counts applied to columns. The ss and ds fractions were concentrated about 10-fold by vacuum dialysis prior to hybridization.

[d] Nitrocellulose filters contained 8 to 10 μg of Ad2 DNA. Reactions were carried out in duplicate. Each reaction vial contained one filter with DNA and one blank filter. Counts per minute bound to blank filters were always less than 0.5% of input counts/min and have been subtracted from the values shown in the table.

[e] The total virus-specific counts per minute on HA were determined from hybridization data. The values given show the distribution, in percent, of virus-specific counts per minute between ss and ds DNA.

[f] In control experiments, 1 μg of [^{3}H]dThd-labeled native viral DNA was added to a pellet of unlabeled infected cells. DNA was isolated exactly as above. Hybridization reactions were omitted.

that digestion of cell lysates by Pronase allowed 90% or greater recovery of intracellular DNA and at least an 85% recovery of encapsidated Ad2 DNA, the efficiency of the present extraction procedure was compared with that of the Pronase digestion method under three sets of experimental conditions (Table 2): (i) the experimental conditions described above which included a 1-h pulse with [^{3}H]dThd beginning 15 h after infection; (ii) the same conditions followed by a cold dThd chase (after the 1-h pulse, cells were washed once and incubated for 3 h in medium containing 100 μg of unlabeled dThd per ml and 10 μg of 2'-deoxycytidine-hydrochloride per ml [3]); and (iii) addition of intact, purified Ad2 virions containing ^{32}P label in DNA to an unlabeled, infected cell pellet immediately before lysis and extraction. In the first instance, total intracellular viral DNA was recovered as efficiently by the extraction procedure as by the Pronase digestion method. In the second instance, the purpose of the 3-h chase was to see if newly labeled DNA moved into forms which were no longer recoverable by the

TABLE 2. *Efficiency of recovery of intracellular viral DNA*[a]

Conditons	Total extracted counts/min ($\times 10^{-5}$)		Total Ad2-specific counts/min[b] ($\times 10^{-5}$)	
	Tris-SDS	Pronase	Tris-SDS	Pronase
1-h Pulse[c]	11.6	11.3	2.2	2.5
1-h Pulse, 3-h chase[d]	12.0	10.7	2.1	2.0
Added ^{32}P-labeled Ad2 virus[e]	6.6	6.5	ND	ND

[a] SDS, Sodium dodecyl sulfate; ND, not determined (hybridization omitted).

[b] Virus-specific counts per minute determined by hybridization to Ad2 DNA as in Table 1. In this experiment the proportion of total label which was virus specific (20%) was somewhat less than that usually found.

[c] Cells infected with Ad2 were pulsed with [^{3}H]dThd for 1 h under conditions described in Table 1, then either carried through the lysis, DNA extraction, and hybridization as described, or digested with Pronase in 0.15 M NaCl, 0.1 M EDTA, 0.3% (wt/vol) SDS for 4 h at 37 C, followed by phenol extraction of DNA and hybridization.

[d] After a 1-h pulse, cells were pelleted, washed, and incubated for 3 h in medium which contained 100 μg of unlabeled dThd per ml, 10 μg of 2'-deoxycytidine-hydrochloride per ml.

[e] Intact, purified Ad2 virions containing ^{32}P-labeled DNA were added to a pellet of unlabeled, infected cells at the time of lysis and digestion. In each case >85% of added counts per minute were recovered.

extraction procedure. Again, nearly all of the viral DNA labeled during the pulse was recovered by the extraction method. Finally, in the last case, even DNA which had been encapsidated into mature virions was efficiently recovered by the method used. It would thus appear that labeled ss Ad2 DNA isolated by the extraction procedure after a 1-h pulse represented a moderate proportion (about 20%) of the total intracellular viral DNA.

Size of DNA fragments. The size of the sheared DNA fragments isolated after extraction and HA chromatography was determined by sedimentation in alkaline sucrose gradients (Fig. 1). DNA in the ss fraction had a mean sedimentation value of 7*S* with a range of 5 to 10*S* (Fig. 1A), whereas the denatured ds DNA fragments had a mean value of 7.5*S* and ranged 4 to 11*S* in size (Fig. 1B). Thus, there was no obvious difference in length distributions of molecules in the ss and ds fractions, suggesting that the presence of DNA in the ss fraction did not depend on the size of DNA fragments generated during extraction.

Strand specificity of ss DNA. The strand specificity of ^{3}H-labeled ss virus-specific DNA obtained by HA fractionation was determined by hybridization to the separated Ad2 H and L DNA strands. Preliminary experiments were done with Ad2 DNA strands applied to nitrocellulose filters (Table 3). Hybridization reactions were carried out with DNA from the ^{3}H-labeled ss and ds fractions isolated in two previous experiments. As shown in the two hybridization experiments, about 40% of ^{3}H-labeled ss DNA counts/min bound to H-strand filters compared to about 30% which bound to L-strand filters. In further experiments hybridizations were carried out in solution as follows: (i) the H- and L-strand pools were each sheared and self-annealed (68 C, 3 h in 0.4 M NaP), adjusted to 0.14 M NaP, and fractionated on HA to obtain highly purified strand-specific DNA; (ii) the respective ss fractions, which were collected in small volumes, were then mixed with ^{3}H-labeled ss DNA and incubated (68 C, 18 h in 0.4 M NaP), and ^{3}H-labeled DNA in hybrids was assayed on HA. As shown in Table 4, under these conditions nearly equal amounts of ^{3}H-labeled ss DNA (44 and 40% of the total virus-specific counts/min) formed hybrids in the presence of Ad2 DNA H or L strands, whereas in the absence of H or L strands a relatively small fraction of the ^{3}H-labeled ss DNA (equivalent to 7% of virus-specific counts/min) was retained by the column. H and L strands, when mixed and incubated, reassociated to the extent of 89%. Therefore, both strands of the Ad2 genome seem to be represented about equally among the ss DNA fragments.

Shift of ss DNA to ds fraction during extended chase. The fate of ss viral DNA was examined in a pulse-chase experiment. After a 1-h pulse with [^{3}H]dThd, infected cells were washed and incubated in medium containing excess unlabeled dThd plus deoxycytidine. Pulse and chase media also contained inorganic ^{32}P to monitor DNA synthesis during both the pulse and chase periods. Figure 2 shows the cessation of [^{3}H]dThd incorporation into DNA during the chase. Incorporation of ^{32}P into alkali-stable counts per minute, however, verifies the continued synthesis of DNA. Table 1 (experiment 2) shows the results of HA separation of ^{3}H-labeled DNA into ss and ds fractions, and the virus specificity of the fractions as determined by hybridization. By the end of 12 h of chase, 75% of the labeled viral ss DNA had

moved into the ds fraction. In addition, there was no loss of total ^3H counts per minute during the chase as might be expected if extensive degradation of DNA was occurring. Therefore, it appears that the ss viral DNA eventually becomes incorporated into ds progeny molecules.

Fractionation of DNA after short pulse. If replication of Ad2 DNA is semiconservative, then after very short pulses most new label should appear in the ds fraction on HA. Table 1 (experiment 3) shows that, after a pulse of 30 s with [^3H]dThd, 94% of virus-specific counts/min were found in the ds fraction.

DISCUSSION

In the present study we describe a method for recovering ss Ad2 DNA synthesized during the period when viral DNA is being replicated. About 20% of total virus-specific DNA synthesized 15 to 16 h after infection was found to be ss (Table 1), and the isolated fragments of this DNA annealed nearly equally with either strand of the Ad2 genome (Table 4).

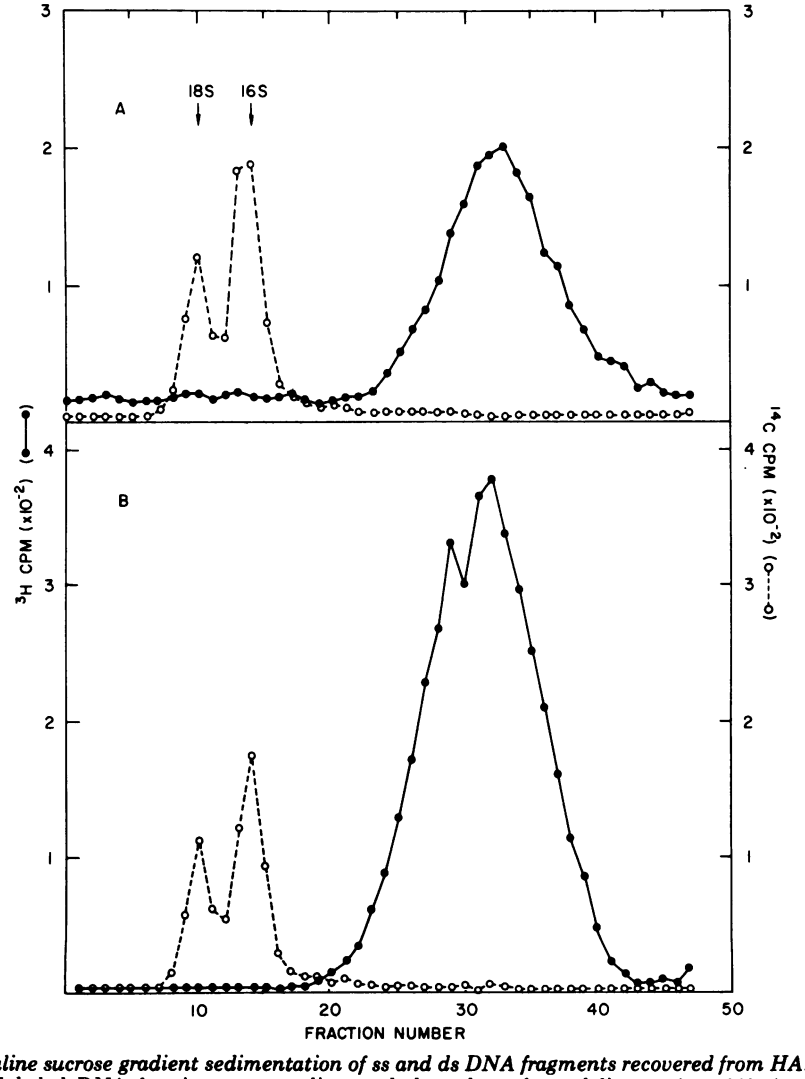

FIG. 1. *Alkaline sucrose gradient sedimentation of ss and ds DNA fragments recovered from HA. Samples of ss and ds ^3H-labeled DNA fractions were sedimented through preformed linear 10 to 30% (wt/wt) sucrose gradients in the SW41 rotor at 40,000 rpm (193,000 × g average) for 17 h at 10 C. Gradients contained 0.7 M NaCl, 0.3 M NaOH, 0.01 M EDTA, and 0.15% (wt/vol) Sarkosyl. ^{14}C-labeled simian virus 40 component II DNA (gift of M. Thoren) was added for size markers. (A) ss fraction; (B) ds fraction.*

TABLE 3. *Hybridization of ss and ds ^{3}H-labeled DNA to separated Ad2 DNA strands*[a]

Expt[b]	Fraction	Input counts/min	Counts/min bound to:		% Counts/min bound to	
			H	L	H	L
1	ss	10,360	4,175	3,298	40	32
1	ds	38,980	9,300	8,600	24	22
2	ss	23,774	9,271	6,648	39	28
2	ds	60,194	12,244	10,295	20	17

[a] ss and ds ^{3}H-labeled DNA were isolated by HA chromatography from Ad2-infected cells after a 1-h pulse with [^{3}H]dThd. Ad2 H strands (1.8 μg) and L strands (1.9 μg) separated using poly(U,G) were applied to nitrocellulose filters in the presence of 5 × SSC (0.75 M NaCl plus 0.075 M sodium citrate).

[b] Experiments 1 and 2 do not correspond to experiments bearing the same numerals in Table 1. In experiment 1 above, blank filters bound between 0.1 and 0.2% of input counts/min. In experiment 2, blank filters bound 0.1% of input counts/min.

TABLE 4. *Hybridization in solution of ^{3}H-labeled ss Ad2-specific DNA to separated strands of Ad2 DNA*[a]

Reaction[b]		% ^{3}H-labeled counts/min in hybrids[c]	% ^{32}P-labeled counts/min in hybrids
^{32}P-Ad2 DNA strand	Ad2-specific ^{3}H-labeled ss counts/min		
H (0.59 μg)	2,702	44	≤4[d]
L (0.36 μg)	2,702	40	≤4[d]
None	2,702	7	
H (0.28 μg) plus L (0.23 μg)	None		89

[a] H and L Ad2 DNA strands were separated using poly(U, G). ^{3}H-labeled ss DNA was isolated by HA chromatography from Ad2-infected cells after [^{3}H]dThd pulse of 1 h.

[b] Final salt concentration was 0.4 M NaP and final volume of each reaction mixture was 1 ml. Virus specificity of ^{3}H-labeled ss DNA was determined by prior filter hybridization as described in Table 1.

[c] Hybrids were assayed by retention on HA.

[d] Because ^{32}P Ad2 DNA strands were in excess, the amount retained in hybrids with ^{3}H-labeled DNA did not exceed 4%.

The possibility that the ss viral DNA is derived from replicating molecules is suggested by the finding that it shifted predominantly to the ds fraction on HA during an extended chase (Table 1). This is consistent with previous reports which present evidence for ss DNA in replicating adenovirus DNA molecules (20, 22). Alternatively, the relatively slow conversion of ss viral DNA to ds molecules could mean that the bulk of this DNA is not primarily involved in DNA synthesis. In addition, it is even possible that the ss DNA was generated by nuclease action either intracellularly or during DNA extraction although this seems very unlikely. If the ss DNA largely originated from replicating molecules its slow conversion to ds might be explained by (i) extensive reinitiations of duplex templates resulting in continued displacement of DNA strands labeled during the

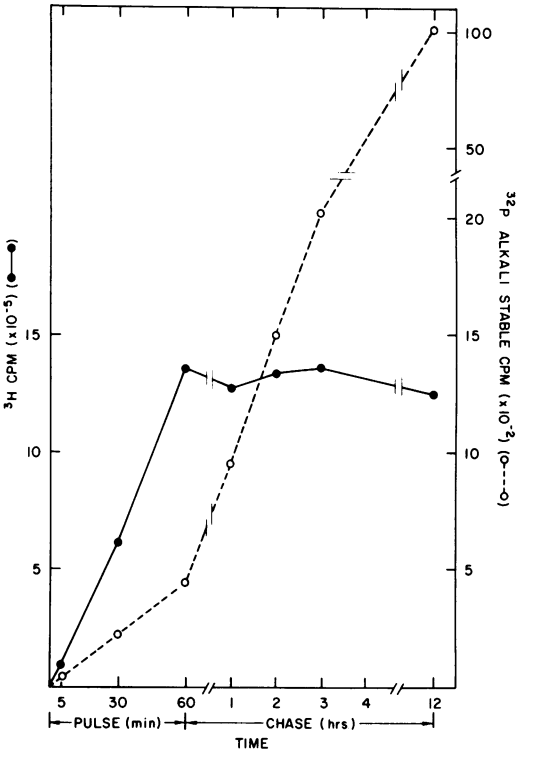

FIG. 2. *Pulse-chase kinetics of incorporation of [^{3}H]dThd and ^{32}P into DNA. A culture of KB cells infected with Ad2 for 15 h was resuspended in phosphate-free Eagle medium containing 2% (vol/vol) regular medium for supplemental phosphate (pulse medium) and pulsed for 1 h with 20 μCi of [^{3}H]dThd per ml and 1 μCi of ^{32}P per ml in the presence of 10^{-5} M FdUrd. Cells were then sedimented, washed in chase medium (pulse medium containing 10^{-5} M FdUrd, 1 μCi of ^{32}P per ml, 100 μg of unlabeled dThd per ml, and 10 μg of 2'-deoxycytidine-hydrochloride per ml) and resuspended in chase medium. At the points shown, 1 ml of cells was removed, washed once, resuspended in 0.5 M NaOH, and incubated overnight at 37 C. Acid-precipitable counts per minute were measured in duplicate.*

pulse period; (ii) a slower conversion of labeled, displaced DNA strands to duplex molecules; or (iii) experimental conditions employed during the chase (e.g., the presence of FdUrd). Although van der Eb (22) found that in Hirt extracts of Ad5-infected cells analyzed in sucrose gradients, virtually all counts in a short pulse shifted to the position of mature DNA after a 30-min chase, he also noted that 20 to 25% of viral DNA still contained ss regions after a 60-min chase when DNA was analyzed by benzoyl-naphthoyl-DEAE-cellulose chromatography.

In two instances, namely the 30-s pulse and the 12-h chase experiments (Table 1), a minimum of 6% ss DNA was detected. The nature of this DNA and the failure to observe an essentially zero value for ss DNA under these two conditions is not clear. It is possible that this fraction represents short, new initiations in the case of the 30-s pulse or, in either case, very small duplex fragments which denature at or below the temperature at which the HA chromatography is performed (60 C). Importantly, mature native Ad2 DNA used in control marker experiments fractionated as greater than 99% ds.

If the observed ss viral DNA does play a role in DNA replication, the finding that this DNA annealed nearly equally with each strand of virion DNA might indicate a conflict with the strand displacement mechanism proposed by Sussenbach et al. (20). They found by hybridization experiments that the same Ad5 DNA strand was always displaced, and by partial denaturation mapping of Y-shaped molecules, that displacement synthesis always started from the same (right) molecular end. However, the presence in replicating molecules of ss DNA representing both Ad2 DNA strands would suggest that either strand is displaced and, therefore, that synthesis can be initiated from either end of the parental duplex molecule. Regarding this possibility, Tolum and Pettersson (21) have also demonstrated that both strands of Ad2 DNA are exposed during replication and, additionally, provided evidence for termination sites of DNA replication at both ends of the viral chromosome. The findings of Garon et al. (5) and Wolfson and Dressler (25) that adenovirus DNA contains an inverted terminal repetition provides a potential structural basis for such a DNA synthesizing mechanism. As a consequence of the inverted repetition the ends of parental duplex molecules would be identical and initiation could begin at either molecular end if this process depended upon a specific terminal sequence (9).

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