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Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization Assay for Replication Origin Deoxyribonucleic Acid of *Escherichia coli*

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Deoxyribonucleic acid (DNA)-DNA hybridization on nitrocellulose filters can be used to assay for replication origin DNA from *Escherichia coli* if the DNA attached to the filters is enriched for the replication origin sequences. Such DNA can be readily isolated from very rapidly growing cells. When low amounts of this DNA were attached to filters, radioactively labeled DNA from the replication origin hybridized 1.7 times as well as radioactive replication terminus DNA. Under identical conditions, radioactively labeled DNA from exponentially growing cells hybridized only 1.3 times as well as radioactive replication terminus DNA. The replication origin, replication terminus, and randomly labeled DNA hybridized with similar efficiencies to filters containing DNA isolated from cells incubated in the absence of required amino acids. This DNA appeared to have all sequences present at equal frequencies. The hybridization assay was used to demonstrate that the DNA synthesized shortly after the addition of amino acids to cells previously deprived of required amino acids was primarily from the replication origin and then rapidly became similar to DNA synthesized by exponentially growing cells.

A deoxyribonucleic acid (DNA)-DNA hybridization assay for replication origin DNA would facilitate studies of the initiation of bacterial chromosome replication in *Escherichia coli*. Transformation can be used to great advantage in studies of initiation in *Bacillus subtilis* (15, 21) because a number of loci are now known which are very close to the replication origin (7). No comparable procedure is available for studies of initiation in *E. coli*. Most analyses of initiation in *E. coli* have utilized the density-transfer techniques developed by Lark and co-workers (10). This procedure has some disadvantages. It is time-consuming, and it is not effective in conditions where a pulse of radioactive precursors can not be quickly "chased" (9). In addition, the density-transfer procedure only demonstrates that the DNA synthesized at a particular time is primarily from the replication origin. If this DNA can be fractionated into several components, it is not possible to determine whether all of it is replication origin DNA.

We have developed a DNA-DNA hybridization assay for replication origin DNA that should be effective in studying initiation in *E. coli* in situations where density-transfer can not be readily used. This procedure should also be useful in some studies with *B. subtilis* if the DNA being investigated is of low molecular weight. The transforming activity of such DNA is severely reduced (2, 4). The rationale of the procedure is as follows. If *E. coli* cells are incubated in the absence of required amino acids, no new replication cycles are initiated and the cycles of replication in progress proceed to completion (10). DNA extracted from such cells should have all genes present at equal frequency, and the ratio of replication origin DNA to replication terminus DNA should be 1/1. If this DNA is attached to nitrocellulose filters (6), radioactively labeled replication origin DNA, replication terminus DNA, and randomly labeled DNA should hybridize to it with similar efficiencies. Conversely, if *E. coli* cells are grown at a very high growth rate, the ratio of replication origin DNA to replication terminus DNA can be as high as 4/1 (5, 18). If DNA extracted from such cells is attached to nitrocellulose filters, radioactively labeled replication origin DNA, replication terminus DNA, and randomly labeled DNA

should hybridize to the DNA on the filter with different efficiencies. The differences should be greatest when the DNA on the filter is not in excess and the genes present at the lowest frequency become saturated while the other genes are still in relative excess. This paper describes our tests of this hybridization assay for replication origin DNA.

MATERIALS AND METHODS

Bacteria. *E. coli* 15 TAU-bar was used for all experiments. The strain required thymine, arginine, uracil, methionine, proline, and tryptophan.

Radiochemicals. ^{3}H-thymine (53 Ci/mmole), ^{3}H-thymidine (47 Ci/mmole), and ^{14}C-thymine (56 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Uniformly labeled ^{14}C-thymidine (520 mCi/mmole) was purchased from Amersham/Searle Corp., Arlington Heights, Ill.

Media. L-broth medium contained 5 g of yeast extract (Difco), 10 g of tryptone (Difco), and 5 g of NaCl per liter of water. The minimal, low-phosphate medium (LP medium) has been previously described (9). This medium was supplemented with (per milliliter): 2 mg of glucose, 2 μg of thymine, 50 μg of arginine, 20 μg of uracil, 50 μg of methionine, 50 μg of proline, and 20 μg of tryptophan. When large volumes (10 to 20 liters) were to be incubated in the absence of a required amino acid, the cells were grown in medium in which the arginine concentration was reduced to 5 μg/ml. For all other incubations in the absence of required amino acids, arginine, proline, and tryptophan were removed from the medium. Membrane filters (type HA, Millipore Corp., Bedford, Mass.) were used to effect washing and transfer of the cells.

DNA isolation. Cells grown in 10 to 20 liters of medium were rapidly chilled with ice and NaN_3 was added to a final concentration of 0.01 M. The cells were collected by centrifugation, washed with ice-cold TV [0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.01 M ethylenediaminetetraacetate, *p*H 8.1], and resuspended in 75 ml TV. Lysozyme was added (300 μg/ml) and the cells were incubated at 37 C for 15 min. Sodium lauryl sulfate was then added (0.5%, w/v) and the lysate was incubated at 65 C for 15 min. Pronase (Calbiochem) was added (400 μg/ml) and incubation was continued at 37 C for 2 hr. Redistilled phenol (75 ml) was added, the mixture was shaken for 20 min and centrifuged, and the aqueous phase was removed. The phenol phase was extracted once with 20 ml of TV and the aqueous fractions were pooled. Two volumes of ethanol were added and the precipitated DNA was collected on a glass rod and dissolved in 25 ml of dilute saline-citrate (11). Concentrated saline-citrate was added (0.1 volume, 10-fold concentrated), and the DNA was incubated at 37 C with 20 μg of pancreatic ribonuclease and 2 μg of ribonuclease-T_1 (Calbiochem, Los Angeles, California) per ml for 45 min. The DNA was then deproteinized repeatedly with chloroform-isoamyl alcohol and precipitated once with ethanol, once with isopropanol, and again with ethanol, as described by Marmur (11).

Preparation of spheroplasts. After a specific labeling or incubation procedure, the cells were immediately diluted with ice-cold TV-CN (TV containing 0.01 M NaCN), centrifuged, and washed twice with TV-CN. The cells were then incubated at a concentration between 5×10^8 and 10^9 cells/ml for 15 min at 0 C in TV-CN containing 3% sucrose (w/v) and 100 μg of lysozyme per ml. For density gradient centrifugation the spheroplasts were incubated with 0.5% Sarkosyl (Geigy Chemical Co., Ardsley, N.Y.) for 15 min at 65 C; for hybridization the spheroplasts were mixed with an equal volume of 0.2 N NaOH.

Density-transfer analysis of replication origin DNA. Cells were grown exponentially for three generations, incubated for 150 min in the absence of arginine, proline, and tryptophan, and labeled with ^{3}H-thymine (1.5 Ci/mmole, 2 μg/ml) from 10 to 22 min after the readdition of the amino acids. The cells were washed and diluted eightfold, grown for three generations, and incubated for 90 min in the absence of amino acids. ^{14}C-thymine (0.62 mCi/mmole, 2 μg/ml) was present at all stages of these incubations. The cells were then transferred to complete medium containing 2 μg of 5-bromouracil per ml in place of thymine, and samples were taken after 27, 33, 39, 45, 53, and 63 min for CsCl density gradient centrifugation. Spheroplasts were prepared and treated with Sarkosyl, and 0.8 ml was vigorously mixed with 1.6 ml of a stock CsCl solution. This was then layered (2.2 ml) on top of 2.2 ml of the stock CsCl solution in a nitrocellulose centrifuge tube. The stock CsCl solution contained 30 g of CsCl and 18 ml of TV. The layered gradients reached equilibrium rapidly (3); centrifugation was conducted for 18 hr at 33,000 rev/min in a Spinco SW50.1 rotor. Figure 2 shows the percentage of the total ^{3}H and ^{14}C radioactivity present in the hybrid density DNA in the different samples.

Hybridization mixture. The crude lysate preparations (spheroplasts plus an equal volume of 0.2 N NaOH) were mixed in the proportions required for a particular hybridization. NaOH (5 N) was added to a final concentration of 0.5 N, and the mixture was hydrolyzed at 94 C for 8 min (19). Preincubation medium containing 5 N HCl sufficient to neutralize the NaOH was then added to give a final DNA concentration of 2 μg/ml. The amount of DNA in the hybridization mixture was calculated from the number of cells used and the amount of DNA per cell during exponential growth or after amino acid deprivation. There was a minimum of 1,500 ^{14}C counts/min and 2,500 ^{3}H counts/min per 0.2 μg of DNA in the hybridization mixture; at least 90% of the DNA was from cells incubated in the absence of arginine, proline, and tryptophan until DNA synthesis ceased. When necessary, nonradioactive DNA obtained from cells incubated for 150 min in the absence of these amino acids was added as one of the components of the hybridization mixture.

DNA-DNA hybridization. The purified DNA was attached to membrane filters as described by Denhardt (6), except the DNA was denatured with 0.2 N NaOH for 10 min and then neutralized, and the DNA filters were heated at 80 C for 2 hr at 1

atmosphere after drying overnight in a vacuum desiccator. It was found important to use freshly prepared filters to obtain maximum hybridization. The filters were preincubated for 5 hr at 65 C in 1 ml of the preincubation medium described by Denhardt with 0.02 M morpholinopropane sulfonic acid, *p*H 7.0, added as a buffer. Hybridization mixture (0.1 ml, 0.2 μg of DNA) was then added, and incubation at 65 C was continued for 18 hr. Each side of the filters was washed with 40 ml of 3×10^{-3} M Tris, *p*H 9.4 (20). The dried filters were counted in 10 ml of a scintillation fluid containing 4 g of Omnifluor (New England Nuclear Corp.) per liter of toluene.

Determination of the efficiency of hybridization. The energy spectra in the scintillation counter of hybridized ^{3}H-DNA and ^{14}C-DNA were lower than those for similar material precipitated with trichloroacetic acid. Consequently, several corrections were required before the radioactivity detected on the hybridization filters could be compared to the radioactivity detected in trichloroacetic acid precipitates of the hybridization mixture. To determine the amounts of ^{3}H-DNA and ^{14}C-DNA in the hybridization mixture, samples (0.1 ml) of the mixture and of preparations containing only ^{3}H-DNA or ^{14}C-DNA were precipitated with trichloroacetic acid and washed on membrane filters with ice-cold water. The dried filters were counted in a Beckman LS233 scintillation counter with the variable discriminators set so that 22 and 78% of the detectable ^{14}C counts/minute were counted in the lower and upper windows, respectively. Only 1.5% of the detectable ^{3}H counts/minute were counted in the upper window, and this carry-over was ignored when the ^{3}H-DNA and ^{14}C-DNA counts/minute present in 0.1 ml of the hybridization mixture were calculated.

The hybridized samples were counted with the same settings on the scintillation counter, along with freshly prepared samples containing only ^{3}H-DNA or ^{14}C-DNA. Between 65 and 75% of the detectable hybridized ^{14}C-DNA counts/minute was counted in the upper window, the amount varying slightly from experiment to experiment. Less than 1% of the detectable hybridized ^{3}H-DNA counts/minute was counted in the upper window, and this carry-over was ignored in further calculations. The data from the hybridized samples were corrected for the carryover of the ^{14}C-DNA into the lower window, and the ^{3}H-DNA counts/minute detected in the lower window were compared with the ^{3}H-DNA counts/minute present in the hybridization mixture to determine the efficiency of hybridization. The ^{14}C-DNA counts/minute detected in the upper window were multiplied by the appropriate correction factor to determine the total ^{14}C-DNA counts/minute, and this was compared to the ^{14}C-DNA counts/minute present in the hybridization mixture to determine the efficiency of hybridization.

Other procedures. The colorimetric assays used to determine the amounts of DNA, ribonucleic acid (RNA) and protein present in the DNA preparations have been previously described (9). In some hybridizations the procedures of Meijs and Schilperoort (13) were used to determine the amounts of DNA fixed to the nitrocellulose filters before and after hybridization. The optical density of cultures was determined in a Bausch & Lomb Spectronic 20.

RESULTS

Preparation of DNA and DNA filters. *E. coli* 15 TAU-bar cells were grown in the presence of limiting arginine (5 μg/ml) with all other nutrients present in excess to obtain DNA from cells incubated in the absence of a required amino acid. The generation time was 60 min, and cell mass abruptly ceased increasing at an optical density of 0.3 at 450 nm. DNA synthesis ceased 60 min later after a 50% increase in DNA, and the cells were harvested after a total of 2 hr of incubation in the absence of mass increase. The DNA was purified as described above, and the DNA was called AA$^-$ DNA. It contained less than 1% RNA and protein.

Cells were grown in L-broth medium to obtain DNA from very rapidly growing cells. The cells grew with a 23-min generation time, and the cells were harvested in log phase at an optical density at 550 nm of 0.2. The purified DNA, which was called L-broth DNA, contained less than 1% RNA and protein.

DNA was attached to nitrocellulose filters by the procedures described by Denhardt (6). When a solution containing the denatured DNA was passed through the filters in the presence of sixfold concentrated saline-citrate, virtually all of the DNA remained attached to the filter. Some of the DNA became detached during incubation at 65 C, as has also been noted by others (1, 17). Table 1 shows there was no significant difference between the retention of L-broth and AA$^-$ DNA, although only 62% of the attached DNA was retained when 1 μg of DNA was added to the filters. We also used the procedures of Meijs and Schilpe-

TABLE 1. *DNA retained on nitrocellulose filters after incubation at 65 C*[a]

DNA added to filter (μg)	L-broth DNA (μg)		AA$^-$ DNA (μg)	
	DNA attached	DNA retained	DNA attached	DNA retained
1	1.0	0.63	1.1	0.68
10	10	7.6	10	7.6
40	37	34	40	32
100	95	81	93	86

[a] L-broth DNA and AA$^-$ DNA were mixed with ^{14}C-DNA and the solutions were filtered through nitrocellulose filters at concentrations between 0.1 and 10 μg/ml. The amounts of DNA attached and retained after 18 hr of incubation at 65 C were determined from the radioactivity present on the filters and the specific activities of the solutions.

roort to extract the DNA from the filters before and after hybridization (13). The amount of DNA was determined by a diphenylamine method (8), and the results were similar to those presented in Table 1 (*unpublished data*).

Preparation of origin and terminus DNA. Amino acid removal and readdition was used to obtain radioactive replication origin DNA for hybridization to the DNA attached to the nitrocellulose filters. Exponentially growing cells were incubated in the absence of arginine, proline, and tryptophan for 150 min, and the amino acids were added back to the medium. Control experiments demonstrated that DNA synthesis resumed after a lag of approximately 20 min (Fig. 1). The radioactive replication origin DNA was obtained by incubating nonradioactive cells with high-specific-activity ^{3}H-thymine (8.5 Ci/mmole, 2 μg/ml) from 10 to 22 min after the readdition of the amino acids. Crude lysates were made from the labeled cells as described in Materials and Methods, and the DNA in the crude lysates was used for the hybridizations. The DNA contained approximately 125,000 counts per min per μg.

A density-transfer experiment was performed to determine whether the labeling procedure labeled the replication origin. The details of the experiment are described in Materials and Methods. Figure 2 shows the ^{3}H-thymine-labeled DNA was preferentially replicated when initiation of replication occurred in medium containing 5-bromouracil. This indicates that the ^{3}H-thymine pulse was incorporated primarily into DNA at or near the replication origin of the chromosome (10).

Amino acid deprivation was also used to

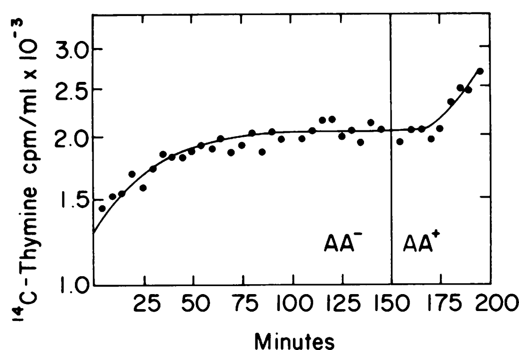

FIG. 1. *Increase in DNA during 150 min of incubation in the absence of arginine, proline, and tryptophan and after the readdition of these amino acids at 150 min. The cells were labeled with ^{14}C-thymine (3.12 μCi/mmole, 2 μg/ml) three generations prior to the amino acid deprivation and during all subsequent incubations.*

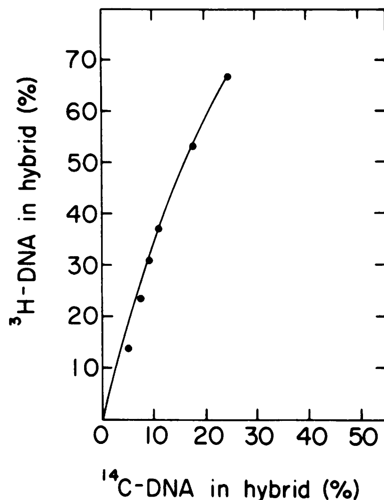

FIG. 2. *Comparison of the replication of DNA uniformly labeled with ^{14}C-thymine and replication origin DNA labeled with ^{3}H-thymine. The previously labeled cells were transferred to complete medium containing 2 μg of 5-bromouracil per ml in place of thymine after 150 min of incubation in the absence of arginine, proline, and tryptophan, and samples were removed for analysis in CsCl density gradients. The figure presents the per cent of the total ^{3}H-DNA and ^{14}C-DNA that was in hybrid density DNA. Details of the experiment are described in Materials and Methods.*

obtain the radioactive replication terminus DNA required for the hybridizations. Exponentially growing cells were transferred to medium lacking arginine, proline, and tryptophan, and after 45 min of incubation the cells were washed and incubated for 15 min in medium in which thymine was replaced by uniformly labeled ^{14}C-thymidine (520 mCi/mmole, 0.2 μg/ml). This procedure labeled only the last DNA synthesized during amino acid deprivation (Fig. 1), which should be enriched for the terminal sequences of the replication cycle. Crude lysates were prepared from the cells, and the DNA contained approximately 17,000 counts per min per μg.

Hybridization assay for replication origin DNA. The basis of the hybridization assay for replication origin DNA is the different efficiencies of hybridization of replication origin and terminus DNA to L-broth DNA. To facilitate a direct comparison of the hybridization efficiencies, a hybridization mixture was made containing ^{3}H-DNA from the replication origin and ^{14}C-DNA from the replication terminus. Precautions were taken to have all genes present at the same frequency in the hybridization mixture. Otherwise, differences in the

efficiencies of hybridization could result from differences in the gene frequencies in the hybridization mixture and not from different gene frequencies in the DNA attached to the filters.

The ^{14}C-thymidine-labeled replication terminus DNA preparation presented no problem in the construction of the hybridization mixture, since the cells had been incubated for 60 min in the absence of the amino acids, and all genes should be present at nearly equal frequencies. This was not true of the ^{3}H-thymine-labeled replication origin DNA. Little net DNA synthesis had occurred by 22 min after the addition of the amino acids (Fig. 1), but some cells had replicated the replication origin (Fig. 2). To insure that the frequency of origin and terminus sequences were as identical as possible, the hybridization mixtures were constructed so that at least 90% of the total DNA came from the ^{14}C-thymidine-labeled cells or from unlabeled cells incubated for 150 min in the absence of the amino acids, and less than 10% of the total DNA came from the ^{3}H-thymine-labeled cells. Consequently, even if every cell had initiated replication at the origin within 22 min after the addition of the amino acids, the ratio of the replication origin DNA to replication terminus DNA should not be greater than 1.1/1. The hybridization mixtures contained at least 2,500 ^{3}H counts/min and 1,500 ^{14}C counts/min in the 0.2 μg of DNA used per hybridization.

Figure 3A shows the results of a hybridization between AA^{-} DNA attached to filters and a hybridization mixture containing ^{3}H-DNA from the replication origin and ^{14}C-DNA from the replication terminus. The efficiency of hybridization increased as the amount of DNA on the filter increased, but the ratio of the efficiencies of hybridization remained constant at approximately 1.0 (Fig. 3C). This constant ratio is to be expected if all genes were equally frequent in the DNA on the filter and in the hybridization mixture. The origin and terminus sequences in the DNA on the filter would become hybridized to the same extent with the DNA from the hybridization mixture.

Figure 3B shows the efficiency of hybridization of the same hybridization mixture to L-broth DNA attached to filters. The efficiency of hybridization of both the ^{3}H-DNA and the ^{14}C-DNA increased as the amount of DNA on the filter increased, but the ratio of the efficiencies of hybridization did not remain constant. This ratio increased to a maximum of 1.7 as the amount of DNA on the filter decreased to 1 μg (Fig. 3C). The increase in the ratio presumably resulted because the origin sequences were more frequent than the terminus sequences in the DNA attached to the filter. The terminus sequences consequently became more saturated than the origin sequences as the amount of DNA on the filter decreased. This decreased the efficiency of hybridization of the terminus sequences more than it decreased the efficiency of hybridization of the origin sequences.

The maximum ratio for the efficiencies of hybridization to L-broth DNA was not 1.7 in all the experiments we conducted, and the ratio obtained with AA^{-}DNA was not always 1.0. However, the quotient of the maximum L-

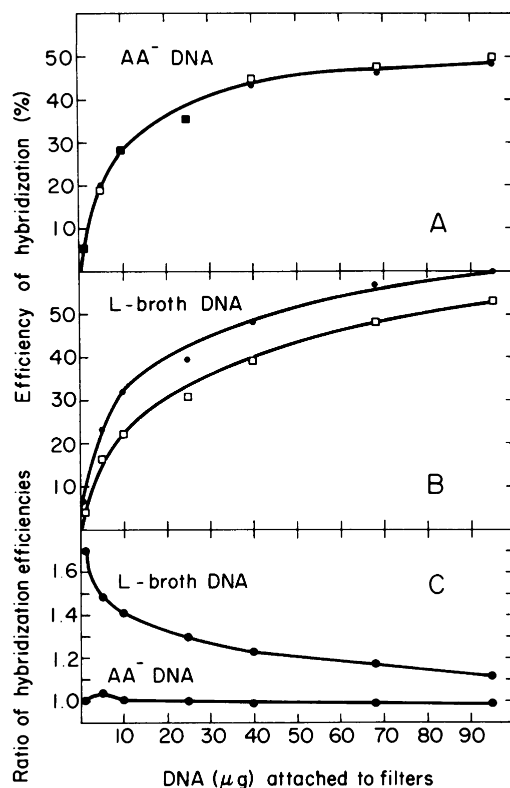

FIG. 3. (*A*) *Efficiency of hybridization of ^{3}H-DNA from the replication origin* (●) *and ^{14}C-DNA from the replication terminus* (□) *to increasing amounts of AA^{-} DNA attached to nitrocelluslose filters. Abscissa shows the amounts of AA^{-} DNA attached to the filters at the beginning of the hybridization. Hybridizations were conducted with 0.2 μg of DNA in 1.1 ml of preincubation medium.* (*B*) *Efficiencies of hybridization of the same hybridization mixture to increasing amounts of L-broth DNA attached to nitrocellulose filters.* (*C*) *Ratio of the efficiency of hybridization of the ^{3}H-DNA to efficiency of hybridization of the ^{14}C-DNA.*

broth ratio divided by the ratio obtained with AA⁻ DNA was always between 1.65 and 1.75. This suggests that the experiment-to-experiment variations (±15%) in the hybridization ratios resulted from an over- or underestimate of the efficiency of hybridization of either the ^{3}H-DNA or ^{14}C-DNA. If the same systematic error was made in calculating the efficiency of hybridization to L-broth DNA and AA⁻ DNA, the error would be canceled when the quotient of the ratios was determined.

There are several places in which a systematic error could have been made in determining the efficiency of hybridization, since it was necessary to use correction factors before the trichloroacetic acid-precipitable radioactivity in the hybridization mixture could be compared to the hybridized radioactive DNA. These factors were required because the energy spectra in the scintillation counter of hybridized ^{3}H-DNA and ^{14}C-DNA were slightly lower than those obtained from the same material precipitated with trichloroacetic acid. As described in Materials and Methods, ^{14}C radioactivity was counted in the scintillation counter with discriminators set so that 78% of the detectable trichloroacetic acid-precipitated radioactivity and 65 to 75% of the detectable hybridized radioactivity was counted in the upper window. The exact proportion of the hybridized radioactivity counted in the upper window varied from experiment to experiment, and the correction factor was changed accordingly. No correction factor was used for the ^{3}H radioactivity, since the spectra for both trichloroacetic acid-precipitated and hybridized ^{3}H-DNA fell entirely within the lower window with the settings used. If some of the hybridized ^{3}H-DNA was not counted because of the decreased spectrum, or if the correction factor used for the hybridized ^{14}C-DNA was in error, this could produce the variations observed. It should be added that even when the ratio for the efficiencies of hybridization on AA⁻ DNA was not 1.0, it was constant with 1 to 100 μg of DNA on the filters. Also, the ratio was always close to 1.0 when the correction factors were based on freshly prepared filters containing only ^{3}H-DNA or ^{14}C-DNA that had been hybridized or precipitated with trichloroacetic acid.

Tests of the hybridization assay. Hybridizations were conducted with radioactive DNA obtained from exponentially growing cells to determine whether the ratio of 1.7 observed in Fig. 3C was indicative of replication origin DNA. Cells were labeled with ^{3}H-thymine (2 Ci/mmole, 2 μg/ml) for one generation, and a hybridization mixture was made with this DNA and ^{14}C-DNA from the replication terminus. Less than 10% of the total DNA in the hybridization mixture was from the exponentially growing cells. Figure 4A shows the ^{3}H-DNA and ^{14}C-DNA hybridized with similar efficiencies to AA⁻ DNA, the ratio of the efficiencies of hybridization being approximately 1.0 (Fig. 4C). The efficiencies of hybridization to the L-broth DNA were not similar, but the ratio of the efficiencies only increased to 1.3 when there was 1 μg of DNA attached to the filters (Fig. 4C). The ratio of 1.7 observed in Fig. 3C indicates that the hybridization procedure is a sensitive assay for replication origin DNA.

A simple experiment was performed to test further the sensitivity of the hybridization assay. Cells were incubated in the absence of arginine, proline, and tryptophan for 150 min, the amino acids were added back to the medium, and the cells were labeled with ^{3}H-thymidine (12.5 μCi/ml, 52 Ci/mmole) for 2 min at 15, 21, 27, 33, 39, 45, and 55 min after the addition of the amino acids. Initiation occurred after the addition of the amino acids (Fig. 2), and the earliest samples should have the highest proportion of the ^{3}H-thymidine incorporated into the replication origin. Hybridization mixtures were made with the ^{3}H-DNA and ^{14}C-DNA from the replication terminus, and the efficiencies of hybridization to filters containing 1 μg of AA⁻ DNA or L-broth DNA were determined. Figure 5 shows the ratio of the hybridization efficiencies (^{3}H-DNA efficiency/^{14}C-DNA efficiency) obtained on AA⁻ DNA and L-broth DNA and the quotient of these ratios. This latter function cancels errors made due to an over- or underestimate of the efficiency of hybridization of the ^{3}H-DNA or the ^{14}C-DNA. Figure 5 shows there was a high rate of initiation during the first 26 min after the addition of the amino acids, and replication then rapidly became randomized.

DISCUSSION

DNA-DNA hybridization on nitrocellulose filters can be used to assay for replication origin DNA if the DNA attached to the filters is enriched for the replication origin sequences. Such DNA can be readily obtained from cells growing in L-broth medium. The 23-min generation time of *E. coli* 15 TAU-bar cells in this medium is a little more than one-half the 40 min required for a replication fork to traverse the chromosome, and the replication origin sequences should be nearly four times as fre-

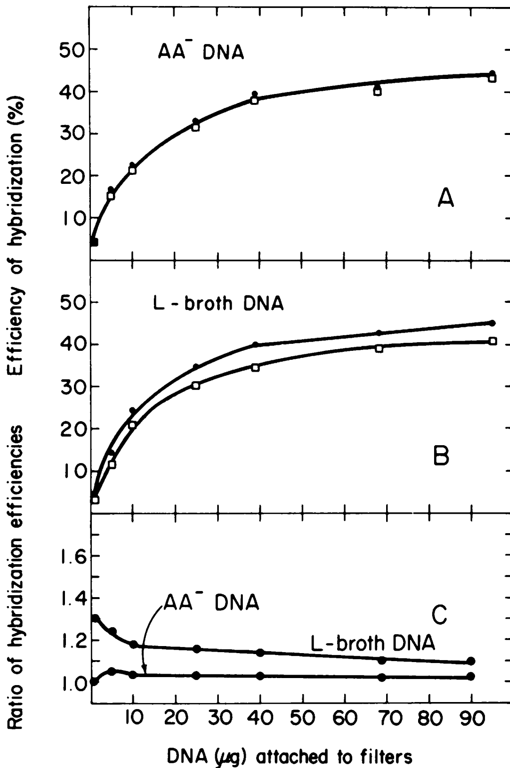

FIG. 4. *(A) Efficiencies of hybridization of ^{3}H-DNA from cells labeled during exponential growth (●) and ^{14}C-DNA from the replication terminus (□) to increasing amounts of AA⁻ dna attached to nitrocellulose filters. Hybridizations were conducted with 0.2 µg of DNA in 1.1 ml of preincubation medium. (B) Efficiencies of hybridization of the same hybridization mixture to increasing amounts of L-broth DNA attached to nitrocellulose filters. (C) Ratio of the efficiency of hybridization of the ^{3}H-DNA to efficiency of hybridization of the ^{14}C-DNA.*

quent as the replication terminus sequences (5, 18). As shown in Fig. 3C, the efficiency of hybridization to L-broth DNA of radioactive DNA from near the replication origin was as much as 1.7 times that of radioactive DNA from near the replication terminus, provided the L-broth DNA attached to the filters was present in limiting amounts. This difference in the efficiencies of hybridization was not observed when the DNA attached to the filters had been extracted from cells incubated in the absence of a required amino acid. The replication origin and terminus sequences should be present at equal frequencies in AA⁻ DNA, and the radioactive replication origin and terminus sequences hybridized to this DNA at equal efficiencies (Fig. 3C).

The ratio of 1.7 for the efficiencies of hybridization of replication origin and terminus DNA was specific for sequences near the replication origin. When exponentially growing cells were labeled with ^{3}H-thymine for one generation and the efficiency of hybridization of this DNA was compared with ^{14}C-DNA from the replication terminus, the ratio of the efficiencies of hybridization to L-broth DNA only reached a maximum of 1.3 (Fig. 4C). The ratio was greater than 1.0 because the exponentially growing cells contained the proximal genes of the replication cycle at a higher frequency than the more distal genes (18), and the specific activities of the genes in the hybridization mixture would have a similar distribution. The ^{3}H-DNA consequently hybridized better than the ^{14}C-DNA to low amounts of L-broth DNA.

As a further test of the sensitivity of the hybridization assay, amino acids were added to cells previously incubated in the absence of required amino acids, and the cells were labeled with ^{3}H-thymidine while initiation was occurring. Hybridization mixtures were made containing the ^{3}H-thymidine-labeled DNA and

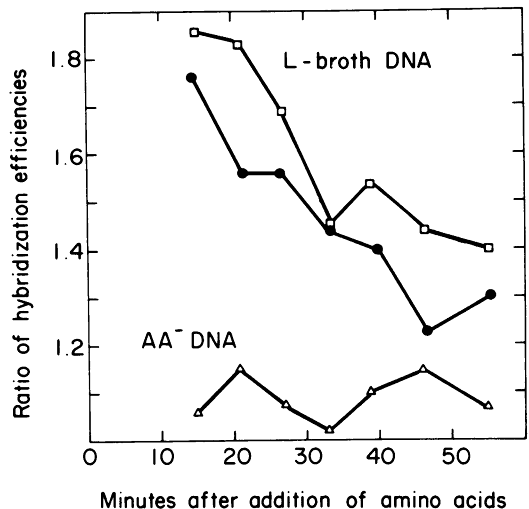

FIG. 5. *Hybridization efficiencies of DNA labeled at intervals after the addition of amino acids to cells incubated for 150 min in the absence of arginine, proline, and tryptophan. The cells were labeled with ^{3}H-thymidine (12.5 µCi/ml, 52 Ci/mmole) for 2 min at the indicated times, and hybridization mixtures containing the labeled cells and ^{14}C-DNA from the replication terminus were made. The mixtures were hybridized to filters containing 1 µg of AA⁻ DNA or L-broth DNA. Ratio of the efficiencies of hybridization (^{3}H-DNA/^{14}C-DNA) on AA⁻ DNA. (△). Ratio of the efficiencies of hybridization (^{3}H-DNA/^{14}C-DNA) on L-broth DNA (□). Quotient of the L-broth ratio/AA⁻ ratio (●).*

^{14}C-DNA from the replication terminus, and the efficiencies of hybridization to filters containing 1 μg of L-broth or AA^- DNA were determined. The relative efficiencies of hybridization were high for the ^{3}H-DNA in the samples obtained during the first 26 min after the addition of the amino acids, and the efficiencies then decreased to the value typical of exponentially growing cells (Fig. 5).

The theoretical maximum for the ratio of the efficiencies of hybridization of replication origin and terminus sequences to L-broth DNA is nearly 4.0. This assumes the cells were in balanced growth when harvested, that the generation time was nearly one-half the time required for a replication fork to traverse the chromosome, that the replication origin and terminus are definite loci on the chromosome, and that the replication origin DNA was not preferentially lost during the DNA isolation. We have been unable to obtain routinely a ratio greater than 1.7 and this is caused by a number of factors:

(i) The theoretical maximum ratio should only be obtained if the DNA attached to the filter is not in excess and all of the replication origin and terminus sequences become saturated with the corresponding DNA in the hybridization mixture. It should have been possible to obtain a ratio greater than 1.7 if less than 1 μg of DNA was attached to the filters, but such hybridizations were not practical. Less than 2.5% of the DNA in the hybridization mixture would have hybridized under these conditions, and DNA with a higher specific activity would have been required to obtain accurate data.

(ii) Another factor which reduced the maximum possible ratio was the relative lack of specificity of the procedures used to label the replication origin and terminus. The ratio of nearly 4.0 should be obtained only when just the most proximal and most distal sequences of the replication cycle were radioactive. The ^{3}H-thymine pulse administered after addition of the amino acids was preferentially replicated during initiation in medium containing 5-bromouracil, but the preferential replication was only approximately threefold (Fig. 2). This indicates that the radioactivity was primarily incorporated near the replication origin, but other sequences could also have become radioactive. This could have resulted from repair processes, or from replication at replication forks at locations other than the replication origin. Also, the pulse was incorporated for 12 min, and chromosomes that initiated replication soon after the addition of amino acids would have had up to 30% of the chromosome labeled.

(iii) The procedure used to label the replication terminus was considerably less specific than that used to label the origin. Depending on when the individual replication cycles terminated during the 15-min pulse with ^{14}C-thymidine, the sequences within the last 38% of the chromosome would have become radioactive to varying degrees. Other sequences also became labeled, since some initiation still occurred in the absence of the required amino acids. This is indicated by the slight increase in DNA after 60 min of incubation (Fig. 1). The major portion of the ^{14}C-thymidine was incorporated in the distal part of the chromosome, however. We have compared the efficiency of hybridization of ^{3}H-DNA from the replication origin with DNA uniformly labeled with ^{14}C-thymine. This latter DNA was obtained by growing cells exponentially for three generations and then incubating them for 150 min in the absence of the required amino acids, ^{14}C-thymine being present at all times. The maximum ratio for the efficiencies of hybridization with L-broth DNA was only 1.4 (*unpublished data*), which was considerably less than the ratio of 1.7 obtained when the ^{14}C-thymidine-labeled replication terminus DNA was used.

Although the hybridization assay is not as sensitive as had been hoped, it has advantages for certain types of studies of initiation. Since it is not necessary to "chase" the radioactive pulse or to grow the cells subsequent to the pulse, the procedure can be used to characterize the DNA synthesized by in vitro replication systems or in vivo after very short pulses. It is often difficult to apply the density-transfer assay to these situations. The hybridization assay can also be used to characterize fractionated DNA, regardless of whether it is of low or high molecular weight or single- or double-stranded. A particular advantage of the assay is that is makes no assumptions about the location of the replication origin, as would an assay using hybridization with F′ episome DNA. Hybridization to episome DNA will probably be the basis of the ultimate assay for replication origin DNA in *E. coli*, but further work is required before the origin and direction or replication will be known with certainty (12, 16).

We have used the assay described in this publication to characterize the very first DNA synthesized after the addition of amino acids to *E. coli* 15 TAU-bar deprived of required amino acids. It was difficult to use density-

transfer techniques to test whether this DNA was from the replication origin, since a short pulse label could not be "chased" quickly enough to label just the DNA being investigated. This DNA separated into several distinct fractions during centrifugation in alkaline sucrose gradients, and we have also used the hybridization assay to test whether the fractions all contain DNA enriched for the replication origin sequences. The results of these experiments will be reported in a subsequent communication.

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