Preparation and Characterization of Highly Radioactive in Vitro Labeled Adenovirus DNA and DNA Restriction Fragments†

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ABSTRACT: A simple microscale procedure is described for the synthesis and purification of high specific activity α-[^{32}P]deoxyribonucleoside 5′-triphosphates (300–1300 Ci/mmol) and their utilization in labeling as little as 56 ng of adenovirus DNA or DNA restriction fragments to specific activities up to 4×10^8 cpm/μg by in vitro repair synthesis ("nick translation"). [^{3}H]DNA of specific activity 1.5×10^7 cpm/μg has also been prepared by nick translation using commercial [^{3}H]thymidine 5′-triphosphate (50 Ci/mmol). These products have been extensively characterized as to reassociation properties. Adenovirus 12 DNA and the EcoRI restriction fragments labeled by this procedure were shown to be identical with in vivo labeled viral DNA by three types of molecular hybridization measurements including (i) copy number determination by reassociation kinetics, of viral DNA in viral transformed cells, (ii) renaturation rate of viral DNA and restriction fragments and agreement with genetic complexity, and (iii) saturation-hybridization of labeled viral DNA with unlabeled restriction fragments. Finally, the distribution of radioactivity in restriction fragments prepared from in vitro labeled DNA showed that viral DNA is uniformly labeled in vitro. These studies represent the first direct demonstration that the in vitro labeling procedure produces sensitive, accurate, and representative DNA probes. Most important, our microscale procedure conserves on expensive nucleoside triphosphates, and valuable (perhaps irreplaceable) viral or tissue nucleic acids. Therefore, this is a versatile method for preparing labeled DNA probes for detecting homologous nucleic acid sequences in small quantities of viral, cellular, or tissue nucleic acids.

Radioactive DNA has been used in numerous types of molecular hybridization studies to characterize the nucleic acid sequences present in cells. For example, labeled viral DNA probes have defined the DNA and RNA in virus infected and transformed cells. The viral DNA probes have usually been labeled in vivo, i.e., by exposing virus infected cells to radioactive precursors; the specific activities obtained, generally 10^6 cpm/μg of DNA or less, are satisfactory for many kinds of studies. For some types of studies, in vivo labeled viral DNA probes are inadequate or cannot be prepared, because probes of the higher specific activity are required to detect viral nucleic acids in low abundance, or because the virus in question cannot be readily grown in cell culture (e.g., papilloma viruses). Recent studies have utilized viral DNA labeled to high specific activities in vitro (Frenkel et al., 1972, 1976; Green et al., 1976a,b, 1977; Jaenisch and Mintz, 1974; Kawai et al., 1973; Mackey et al., 1976; Maniatis et al., 1976; Nonoyama and Pagano, 1973; Shaw et al., 1975). However, in most cases these in vitro labeled probes, which offer many advantages over in vivo labeled probes, have not been extensively characterized regarding uniformity of labeling and reassociation properties.

One in vitro labeling procedure, based on the "nick translation" reaction of DNA polymerase (Kelly et al., 1970), has been described recently by Rigby et al. (1977). In this procedure, single-stranded "nicks" are introduced into DNA by the action of DNase I; these nicks are then repaired by *Escherichia coli* DNA polymerase I in the presence of one or more highly radioactive deoxyribonucleoside 5′-triphosphates, to produce DNA with specific activities in excess of 10^8 cpm/μg (*E. coli* DNA polymerase I binds at single-stranded nicks in a DNA molecule and in the presence of the four dNTPs extends the 3′ primer terminus with simultaneous hydrolysis of the preexisting strand by the 5′→3′ exonuclease activity of the polymerase [Kelly et al., 1970]). As described in this communication, we have modified and adapted this procedure to microscale in order to label small quantities of adenovirus DNA (56 ng) to high specific activities (4×10^8 cpm/μg of DNA) while conserving both valuable DNA samples and costly high specific activity α-[^{32}P]deoxyribonucleoside 5′-triphosphates (1300 Ci/mmol). The viral DNA product is extensively characterized as to size, fidelity, uniformity of labeling, and is compared with in vivo labeled DNA and DNA restriction fragments in reassociation kinetic and hybridization experiments.

Materials and Methods

Reagents. [^{32}P]Orthophosphoric acid, carrier free, was obtained from New England Nuclear Corp. and was suitable for use without further purification. Dimethyl sulfoxide was distilled twice under reduced pressure and stored over CaH_2. Triethylamine (Matheson, Coleman and Bell), acetonitrile, and trichloroacetonitrile were distilled from CaH_2 prior to use and stored over CaH_2.

Stock solutions of 1 M triethylamine bicarbonate were prepared by dissolving triethylamine in water at 0 °C and slowly bubbling high purity CO_2 through the solution until the pH was 7.5–8.0. TEAB[1] was stored at 4 °C in tightly stoppered amber glass bottles.

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[1] Abbreviations used are: Ad, adenovirus; C_0t, parameter used in measuring reassociation equal to the product of the DNA concentration in mol L^{-1} and time in seconds; DEAE, diethylaminoethyl; dNTP, deoxyribonucleoside 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; PEI, polyethylenimine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); QAE, diethyl(2-hydroxypropyl)aminoethyl; TEAB, triethylammonium bicarbonate; Tris, tris(hydroxymethyl)aminomethane.

Enzymes. The nucleotide kinase preparation was purified essentially as described by Bishop et al. (1971). Streptomycin supernatant fluids (Richardson et al., 1964) were fractionated by ammonium sulfate precipitation and adsorption on alumina C_γ gel. The final ammonium sulfate precipitate was dissolved in 10 mM Tris-HCl (pH 8.0) and dialyzed overnight against 10 mM Tris-HCl (pH 8.0)–0.4 M NaCl. The dialyzed preparation was stored at −20 °C and has retained its original activity for 2 years.

E. coli DNA polymerase I used in early experiments was the generous gift of Dr. A. Kornberg. Later preparations were prepared as follows. *E. coli* B ¾ log phase cells were lysed in a Manton-Gaulin disintegrator at 0 °C under 8000–9000 psi in 50 mM Tris–1 mM EDTA (pH 7.5). The cell lysate was fractionated as described by Jovin et al. (1969). Fraction VII DNA polymerase, specific activity 14 000 units/mg, was stored in liquid nitrogen.

Restriction endonuclease R.*Eco*RI was prepared from *E. coli* RY13 as described by Mulder and Delius (1972) and stored at −20 °C. S_1 nuclease was prepared from *Aspergillus oryzae* α-amylase as described previously (Wold et al., 1976).

Pancreatic DNase I (Worthington Biochemical Corp., electrophoretically purified) was dissolved in 10 mM HCl at a concentration of 1 mg/mL and stored in 20-μL aliquots at −20 °C until used. An aliquot of DNase was thawed and diluted with activation buffer (10 mM Tris (pH 7.5)–5 mM $MgCl_2$–1 mg/mL bovine serum albumin) to 100 μg/mL and incubated 2 h at 0 °C (Rigby et al., 1977). Immediately prior to use the DNase was further diluted to 100 ng/mL or 25 ng/mL in activation buffer.

Cell and Viral DNA. Ad12 (strain Huie, plaque 9) and Ad2 (strain 38-2, plaque 4) were grown and purified as previously described (Green and Piña, 1963, 1964; Green et al., 1976a; Piña and Green, 1969). Viral DNA was isolated and purified by the method of Green and Piña (1963, 1964). Ad12 transformed hamster cells (HE C19) were grown as described by Fujinaga and Green (1966) and the DNA was purified as reported previously (Green and Piña, 1963). DNA was isolated from hamster tumors as described elsewhere (Green et al., 1977). Endonuclease R.*Eco*RI fragments of Ad12 DNA were prepared and purified as described previously (Green et al., 1976a).

Synthesis of α-[^{32}P]Deoxyribonucleoside 5′-Triphosphates. At the beginning of these studies, attempts to utilize commercial α-[^{32}P]deoxyribonucleoside 5′-triphosphates for in vitro labeling of DNA often failed due to an known impurity in the triphosphate preparation. To provide more dependable substrates at much higher specific activities than commercially available, α-[^{32}P]deoxyribonucleoside 5′-triphosphates were prepared by a modification of the method of Symons (1969). A mixture containing 5–8 μmol of deoxyribonucleoside, 20 mCi of ^{32}P as H_3PO_4, 15–75 nmol of H_3PO_4, and 50 μL of triethylamine in a 5-mL pear shaped flask was evaporated four times from 2–3 mL of anhydrous acetonitrile; air was admitted slowly to the flask through a drying tube. The anhydrous solid was immediately dissolved in 100 μL of anhydrous dimethyl sulfoxide; 1 μL each of anhydrous triethylamine and anhydrous trichloroacetonitrile were added and the flask was tightly stoppered. The reaction mixture was incubated at 37 °C for 60 min, and the reaction was stopped by the addition of 1.0 mL of distilled water. The reaction mixture was separated by chromatography on QAE-Sephadex as follows. QAE-Sephadex Q-50 was allowed to swell in 1 M formic acid, washed several times with 1 M formic acid, then with water, and stored at 4 °C until used. The QAE-Sephadex Q-50 column (0.4 × 3 cm) was poured and washed with water. The diluted reaction mixture was applied to the column and washed with at least 10 mL of distilled water to remove unreacted deoxyribonucleoside and dimethyl sulfoxide. The mixture of ^{32}P-labeled 3′- and 5′-deoxyribonucleoside monophosphates was eluted in 2–3 mL of 0.1 N formic acid. Unreacted [^{32}P]H_3PO_4 remained on the column and was discarded. The monophosphate mixture was evaporated to dryness 4–6 times from distilled water to remove the formic acid.

α-[^{32}P]Deoxyribonucleoside 5′-triphosphates were prepared by the enzymatic phosphorylation of the deoxyribonucleoside 5′-monophosphates with a mixture of nucleotide kinases from *E. coli.* To the dried mixture of [^{32}P]monophosphates in a 10-mL pear shaped flask was added 50 μL of kinase reaction buffer (0.1 M Tris (pH 8.0), 20 mM $MgCl_2$, 7 mM KCl, 15 mM ATP, and 0.05 mg/mL bovine serum albumin) and 6 μL of nucleotide kinase. The minimal amount of kinase that yielded maximal amounts of triphosphate was determined experimentally for each kinase preparation. The reaction mixture was incubated at 37 °C and the progress of the reaction was followed at 30-min intervals by ascending thin-layer chromatography on polyethylenimine-cellulose in 0.4 M NH_4HCO_3. Markers were used to locate mono-, di-, and triphosphates which were cut out and radioactivity was determined by liquid scintillation counting. When the yield of triphosphate reached a maximum (30–60%), 1 mL of 0.05 M TEAB was added and the reaction cooled to 0 °C. The triphosphate was purified by ion-exchange chromatography on DEAE-Sephadex as follows. DEAE-Sephadex A-50 was allowed to swell in 1 M TEAB. The resin was washed several times with 1 M TEAB, then with distilled water, and stored at 4 °C until use. A 1.0 × 15 cm DEAE-Sephadex A-50 column was poured and equilibrated in 0.05 M TEAB. The diluted reaction mixture was loaded onto the column, washed with 5 mL of 0.05 M TEAB, and eluted with a 200-mL 0.05–0.40 M TEAB linear gradient. The triphosphate peak eluted at 0.2–0.3 M TEAB (depending on the specific deoxyribonucleoside triphosphate) and was pooled and desalted by repeated evaporation from distilled water. After desalting, the purified triphosphate was stored in 50% ethanol at −20 °C. The final product (dATP or dCTP) was >95% triphosphate as determined by PEI-cellulose chromatography. α-[^{32}P]-dNTPs could be utilized for in vitro labeling for 4–10 days with incorporation rate decreasing with time. Repurification of the triphosphate on DEAE-Sephadex as described above restored the original incorporation rate, presumably by removal of an unknown decay product that inhibited the nick translation reaction.

Synthesis and Purification of in Vitro ^{32}P-Labeled DNA and DNA Restriction Enzyme Fragments. Viral DNA and DNA restriction enzyme fragments were labeled by a micro modification of the method described by Rigby et al. (1977) utilizing the nick translation reaction of *E. coli* DNA polymerase (Kelly et al., 1970). Nick translation of viral DNA was carried out in 4-μL volumes. One or two α-[^{32}P]dNTPs or [^{3}H]dNTPs (0.1 nmol) were evaporated to dryness in a 6 × 50 mm glass tube in a vacuum desiccator over Drierite. The reaction mixture (3 μL) containing 0.4 nmol of each of the unlabeled dNTPs, 66.7 mM potassium phosphate (pH 7.5), 6.7 mM $MgCl_2$, and 56 ng of DNA was added to the dried α-[^{32}P]dNTPs at room temperature followed by 0.1 μL of H_2O and 0.4 μL of activated DNase (25 ng/mL with α-[^{32}P]dNTP or 100 ng/mL with [^{3}H]dNTP). The mixture was maintained at room temperature for 60 s and immediately cooled in an ice–water bath. *E. coli* DNA polymerase I (0.5 μL, 7 units) was added and the mixture incubated at 14 °C. The labeling

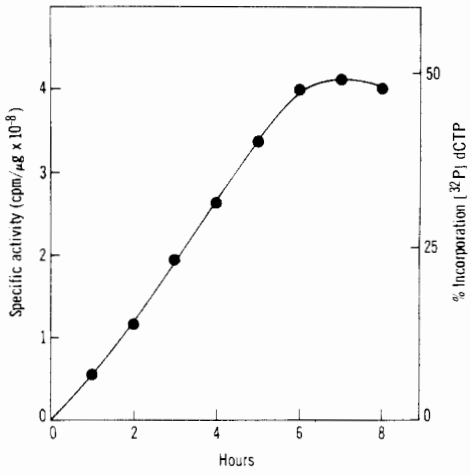

FIGURE 1: Time course of in vitro labeling of Ad12 DNA. Ad12 DNA was labeled by nick translation using [^{32}P]dCTP (500 Ci/mmol) and the reaction was assayed for ^{32}P incorporation into acid-insoluble material as described in Materials and Methods.

reaction was followed by determining acid-precipitable radioactivity as described below. Aliquots (0.1 μL) removed at 1-h intervals were added to 100 μL of 0.01 M EDTA (pH 7.0) and precipitated with 150 μL of cold 10% trichloroacetic acid in the presence of 100 μg of calf thymus DNA carrier at 0 °C for 10 min. After addition of 300 μL of cold water, the mixture was centrifuged at 800*g* for 10 min, the supernatant removed, and the precipitate dissolved in 100 μL of 0.2 M NaOH. DNA was precipitated with 3 mL of cold 10% Cl_3CCOOH, collected on Schleicher and Schuell B6 filters, and counted in a toluene-based scintillation fluid.

The reaction was terminated at 30–60% substitution of dNTP by addition of 100 μL of 0.1 M EDTA (pH 7.0) containing 500 μg/mL of calf thymus DNA and heating at 68 °C for 10 min. Labeled DNA was separated from unincorporated dNTPs by gel filtration on a Sephadex G-50 column (0.8 × 20 cm) using 10 mM Tris (pH 7.5), 1 mM EDTA, and 10 mM NaCl. Radioactive fractions eluting at the void volume were pooled and stored at 4 °C.

Determination of Size of Nick Translated Products by Alkaline Sucrose Density Gradient Centrifugation. Samples of the nick translated products were loaded on 3.6-mL linear 5–20% sucrose gradients containing 0.2–0.8 M NaOH and 0.8–0.2 M NaCl and 2 mM EDTA and centrifuged in a Beckman SW60 rotor 6.5 h at 60 000 rpm at 15 °C. The gradients were fractionated and counted. Size was determined relative to an internal marker (sheared Ad2 [^{14}C]DNA of known size) by the method of Studier (1965).

Hybridization Conditions. Hybridizations were performed in 0.72 M NaCl, 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes) (pH 6.7), 1 mM EDTA, 0.05% sodium dodecyl sulfate. Reaction mixtures (5–100 μL) were sealed in siliconized capillaries and incubated at 68 °C. After incubation samples were cooled immediately to 4 °C and the fraction of double-stranded DNA was determined by batchwise elution from hydroxylapatite (Green et al., 1976a) or by S_1 nuclease digestion (Green et al., 1976b).

Results

Labeling Ad DNA by the Nick Translation Reaction. [^{32}P]deoxyribonucleoside monophosphates were synthesized by the condensation of [^{32}P]H_3PO_4 with the unblocked deoxyribonucleoside in the presence of triethylamine and trichloroacetonitrile. Under these conditions the yield of monophosphate is 70–95%; the product is a mixture of the 3'-monophosphate (35%) and the 5'-monophosphate (65%) (Symons, 1969). Separation of these isomers is not necessary since the 3' isomer is not phosphorylated further in the subsequent enzymatic reaction and therefore does not contaminate the final triphosphate product.

α-[^{32}P]Deoxyribonucleoside 5'-triphosphates were prepared by the enzymatic phosphorylation of the deoxyribonucleoside 5'-monophosphates using a nucleotide kinase mixture from *E. coli* (Bishop et al., 1971). The yields for dATP and dCTP varied from 30 to 60% in 2–4 h; very little diphosphate was produced. DEAE-Sephadex chromatography of the reaction mixture allowed excellent separation of the mono-, di-, and triphosphates. The pooled triphosphate peak was >95% triphosphate as determined by PEI-cellulose thin-layer chromatography. The purity of the TEAB used was critical to the functioning of the product in nick translation. α-[^{32}P]dNTPs of specific activity 300–1300 Ci/mmol have been prepared routinely by this procedure; higher specific activities should be possible.

Using α-[^{32}P]dNTPs of specific activity 600 Ci/mmol, Ad2, 5, 7, and 12 DNA have been labeled routinely to 4×10^8 cpm/μg; this represents about 40% substitution of radioactive dNTP into DNA. Commercial α-[^{32}P]dNTP and [^{3}H]dNTPs are presently available at specific activities up to 200 Ci/mmol and 90 Ci/mmol, respectively and can be utilized for in vitro labeling of DNA; in our experience, it has been often necessary to repurify commercial dNTP preparations by DEAE-Sephadex chromatography in order to permit adequate reaction rates. Using commercial [^{3}H]dTTP (50 Ci/mmol), Ad [^{3}H]DNA has been prepared at 1.5×10^7 cpm/μg (60% substitution). Figure 1 shows a typical time course of the reaction, which was linear for 6 h. Maximum incorporation of radioactive dNTP occurred at 2 to 10 h, depending upon the purity of the preparation of radioactive dNTP.

Size and Hybridization of in Vitro Labeled DNA to Homologous Viral DNA. In the presence of excess homologous DNA, in vitro labeled DNAs generally hybridized 92% to 98%. Usually less than 3% of denatured in vitro labeled DNA were retained on hydroxylapatite as duplex DNA. Occasional preparations of [^{3}H]dTTP or [^{32}P]dNTPs have produced DNA products that appear to contain up to 30% double-stranded DNA after denaturation ("snap back"). The reason for "snap back" is unknown, but has been suggested to reflect a loss of 5'→3' exonuclease activity of the polymerase with subsequent template switching (Rigby et al., 1977; Kornberg, 1974).

Since hybridization rates depend critically on the size of the DNA after denaturation, all products were routinely analyzed by centrifugation on alkaline sucrose density gradients. The sedimentation profile of in vitro labeled Ad2 [^{32}P]DNA is shown in Figure 2. The average size of denatured nick translated products depended on the DNase concentrations as expected (Table I). The products labeled with [^{3}H]dNTPs were consistently larger than those labeled with [^{32}P]dNTPs using the same DNase concentration; the reason for this is unknown, but may possibly be due to additional breaks in DNA introduced by the high levels of ^{32}P during nick translation.

Size and hybridization characteristics of in vitro labeled [^{3}H]DNA probes remained constant for up to 9 months. In vitro labeled [^{32}P]DNA probes of specific activity $>10^8$ cpm/μg were relatively stable for approximately 7–8 days after which time degradation becomes increasingly evident as indicated by decreased average size of the probe and a correspondingly lower rate of reassociation.

The Fidelity of Nick Translated Probes. It is important to

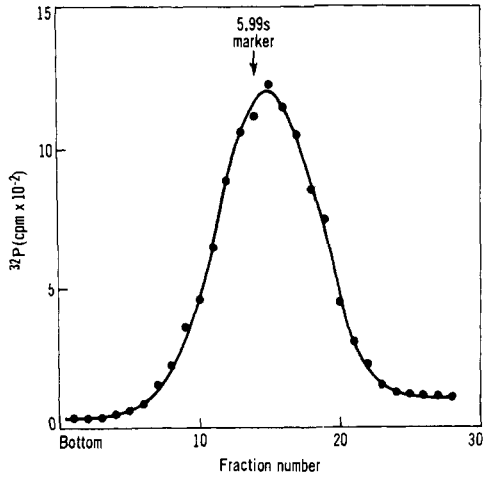

FIGURE 2: Velocity sedimentation of in vitro labeled Ad2 [^{32}P]DNA in 5–20% alkaline sucrose gradient. Ad2 [^{32}P]DNA was centrifuged at 60K for 6.5 h at 15 °C in SW60 rotor with a calibrated [^{14}C]DNA marker as described in Materials and Methods. Fractions were collected from the bottom of the gradient and radioactivity was measured.

TABLE I: Size of in Vitro Labeled DNA Probes.

Experiment	Isotope	DNase concn (ng/mL)	Average size[a] Length (nucleotides)	*S* value
1	^{3}H	10	290–420	5.06–5.87
2	^{3}H	5	570–800	6.63–7.59
3	^{3}H	2.5	1100–1500	8.62–9.76
4	^{32}P	10	100–140	3.31–3.78
5	^{32}P	5	230–280	4.61–4.99
6	^{32}P	2.5	390–490	5.70–6.24

[a] Size of DNA in peak fraction. Each experiment includes size determinations for 3–6 Ad DNA probes. Conditions for in vitro labeling of DNA and for determination of size by alkaline sucrose density gradient centrifugation are described in Materials and Methods.

show that the in vitro repair reaction produces uniformly labeled DNA identical in renaturation characteristics as the same DNA labeled by in vivo techniques. For this purpose, we labeled Ad12 DNA and the 6 Ad12 *Eco*RI DNA restriction fragments both in vitro as described here and in vivo as previously reported (Green et al., 1976a) and compared the renaturation properties of these two sets of products by several methods. First, we assessed the ability of in vitro labeled probes to determine copy number of viral DNA in viral transformed cells. We have previously shown that the Ad12 transformed hamster cell line, HE C19, contains 8–11.5 copies of each of the six Ad12 *Eco*RI DNA restriction fragments (Green et al., 1976a). Reassociation kinetic studies with Ad12 DNA are shown in Figure 3; similar results were obtained with Ad12 *Eco*RI restriction fragment E. The same $C_0t_{1/2}$ values were determined for the reassociation of in vitro and in vivo probes, 5×10^{-3} mol s L^{-1} for Ad12 DNA and 3×10^{-4} mol s L^{-1} for fragment E. The accelerations of reassociation using the two in vitro labeled DNA probes were identical with those with in vivo labeled probes; we estimate the HE C19 cells contain 8 genome equiv of Ad12 DNA and 9.5 equiv of fragment E (Green et al., 1976a). In reconstruction experiments, addition of 2.5 and 12.5 copies of Ad12 DNA produced the same acceleration of reassociation with in vivo and in vitro probes consistent with the equivalence of these probes in annealing reactions (Figure 3).

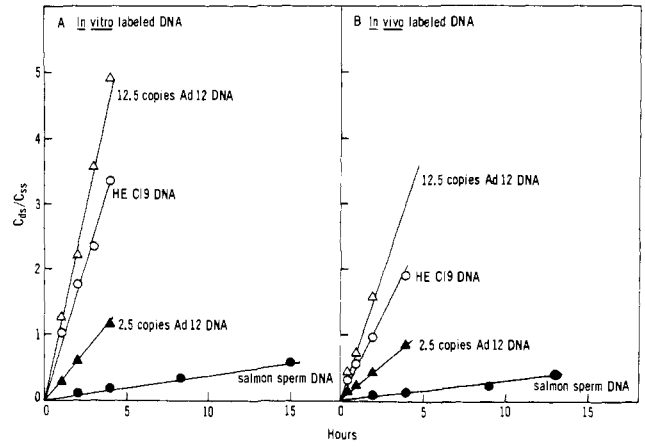

FIGURE 3: Reassociation of in vitro and in vivo labeled Ad12 DNA in the presence of Ad12 transformed cell (HE C19) DNA. Ad12 DNA was labeled in vivo and in vitro as described in Materials and Methods. In vitro labeled Ad12 DNA (1.5×10^{-2} μg/mL, 1.0×10^{8} cpm/μg) was 250–450 nucleotides in length and in vivo labeled Ad12 DNA (1.1×10^{-2} μg/mL, 2.5×10^{7} cpm/μg) was reduced to 400 nucleotide length fragments by sonication. Labeled Ad12 DNA (1300–7500 cpm) in 5 μL was denatured and reannealed under hybridization conditions as described in the text, in the presence of 5 mg/mL of either salmon sperm DNA, HE C19 DNA, salmon sperm DNA plus 0.345 ng (2.5 copies) of unlabeled Ad12 DNA, or salmon sperm DNA plus 1.7 ng (12.5 copies) of unlabeled Ad12 DNA. At the indicated times, the reaction tubes were chilled in ice and stored at 4 °C for later analysis. The maximum percentage of labeled DNA that could form a duplex (generally 92–98%) was determined in a control reaction with added unlabeled Ad12 DNA annealed to C_0t values of over 300 times the expected $C_0t_{1/2}$ for Ad12 DNA (5×10^{-3} mol s L^{-1}). The data were corrected by a computer program (M.R.G., unpublished data) for scintillation counter background, systematic hydroxylapatite errors (about 1% retention and 1% cross over), zero-time duplex DNA (less than 5%), and inability to drive the reaction to completion (92–98% duplex).

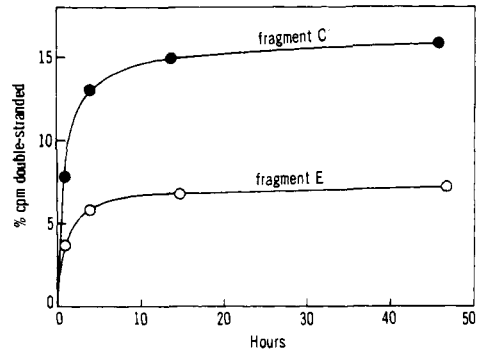

FIGURE 4: Saturation-hybridization of in vitro Ad12 [^{3}H]DNA with Ad12 DNA restriction fragments. In vitro labeled Ad12 [^{3}H]DNA (500 cpm in 50 μL) was denatured and reannealed under standard hybridization conditions in the presence of 60 ng/mL of Ad12 EcoRI C fragment and 6 mg/mL of calf thymus DNA or 27 ng/mL of Ad12 EcoRI E fragment and 6 mg/mL calf thymus DNA. Samples were removed at indicated times and the duplex DNA fraction was determined by hydroxylapatite chromatography.

Second, we showed directly that nick translated products are uniformly labeled by performing saturation-hybridization experiments with nick translated Ad12 DNA and excess unlabeled Ad12 DNA restriction fragments. If the Ad12 DNA is uniformly labeled, then the fraction of the labeled DNA saturated by the unlabeled restriction fragment should be the same as the fraction of the genome the fragment represents. As shown in Figure 4, fragment C, representing 16.3% at the left end of the Ad12 genome, and fragment E, representing 7.2% near the middle of the Ad12 genome (Mulder et al., 1974), saturate 15.8% and 7.2% of nick-translated Ad12 DNA,

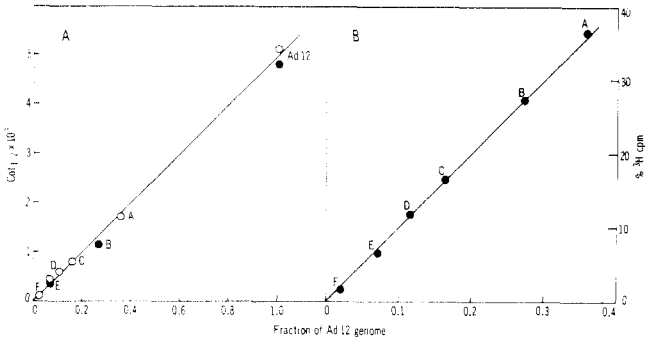

FIGURE 5: Renaturation rate and distribution of radioactivity of in vitro labeled Ad12 EcoRI restriction fragments as a function of genetic complexity. (A) Relationship between genetic complexity (fraction of Ad12 genome) and renaturation rate ($C_0t_{1/2}$) of Ad12 EcoRI restriction fragments. Reassociation kinetic analysis of in vivo labeled Ad12 EcoRI fragments A, C-F (○) and in vitro labeled Ad12 EcoRI fragments B and E (●) were set up as described in the legend to Figure 3 with labeled fragments in the presence of 5 mg/mL of salmon sperm DNA. $C_0t_{1/2}$ values were defined by a C_{ds}/C_{ss} value of 1. The amounts of labeled DNA calculated from the specific activity of the probe and from the observed acceleration in reconstruction reactions with added Ad12 DNA were in good agreement. No corrections were made for the fragment lengths of the various labeled DNA preparations. (B) Distribution of radioactivity in EcoRI restriction fragments of in vitro labeled Ad12 [^{3}H]DNA. Ad12 DNA was labeled with [^{3}H]dTTP in vitro to a specific activity of 9×10^6 cpm/μg. The reaction was stopped by the addition to 100 μL of 0.1 M EDTA (pH 7.0), cooled to 0 °C, and purified on Sephadex G-50 after the addition of 40 μg of unlabeled Ad12 DNA. Approximately 80 000 cpm of Ad12 [^{3}H]DNA containing 5 μg of unlabeled Ad12 DNA carrier was digested with EcoRI as described in the text. Fragments were separated by slab gel electrophoresis, stained with ethidium bromide, and visualized under UV light; visible bands (fragments A-F) were cut out and radioactivity was measured in aqueous scintillation fluid. Data for the fraction of the viral genome represented by each fragment were obtained from Mulder et al. (1974).

respectively. Again, these results suggest that Ad12 DNA is uniformly labeled by our in vitro procedure.

Third, we examined the relationship between the renaturation rate of nick translated probes and their genetic complexity (number of base pairs present in nonrepeated sequences). Since DNA sequences renature at a rate that is proportional to their initial concentration, $C_0t_{1/2}$ values were proportional to the genetic complexity of the DNA species (Britten and Kohne, 1968; Doty et al., 1960; Wetmur and Davidson, 1968). Ad12 DNA and the Ad12 *Eco*RI DNA restriction fragments provide a set of DNA molecules with well-characterized genetic complexities. Endonuclease R. *Eco*RI cleaves Ad12 DNA into six unique fragments with the order C-D-B-E-F-A on the linear viral genome (Mulder et al., 1974; H. Delius and C. Mulder, personal communication). Figure 5A illustrates the relationship between genetic complexity and renaturation rate for in vivo and in vitro labeled Ad12 DNA fragments. Both labeling procedures produced probes that renature at rates predicted by the size of the viral DNA species, suggesting that in vitro labeled DNA is uniformly labeled.

In a final experiment, we approached the question of the uniformity of labeling using a physical analysis of the labeled DNA products. Ad12 DNA was labeled with [^{3}H]dTTP in vitro to a specific activity of 9×10^6 cpm/μg (approximately 36% substitution) and cleaved with endonuclease R.*Eco*RI in the presence of unlabeled Ad12 DNA. The six restriction fragments were isolated and the fraction of radioactivity in each fragment was determined. If the Ad12 DNA was uniformly labeled with ^{3}H, then the percentage of label in each restriction fragment should be in agreement with the percentage of the viral genome which the fragment represents. As shown in Figure 5B, this agreement is excellent.

Discussion

We have adapted the method of Rigby et al. (1977) to a microscale and utilized high specific activity deoxyribonucleoside 5′-triphosphate to prepare radioactive DNA and DNA restriction fragments of specific activities as high as 4×10^8 cpm/μg. This micro method permits the preparation of high specific activity radiolabeled DNA while conserving valuable DNA samples and high specific activity radioactive substrates. We have conducted extensive experimentation to show that the *in vitro* labeled DNA probes are uniformly labeled and that they hybridize identically with in vivo labeled DNA.

In vitro labeling of DNA offers several advantages over conventional in vivo labeling procedures. First, DNA of at least one order of magnitude higher specific activity can be obtained. Therefore hybridization reactions can be established in which the probe undergoes a minimal amount of self-annealing (because the probe is at a very low concentration), and requires as little as 30 μg of valuable cell or tissue DNA (e.g., from tissue biopsies) to determine the presence or absence of viral DNA sequences in the sample. Second, any DNA can be labeled in vitro, regardless of whether adequate tissue culture systems have been established. For example, we have labeled whole DNA and restriction fragments from 12 human Ads, herpes virus 1 and 2, human plantar wart virus, BK virus, SV40, and human sperm. Third, in vitro methods allow for the initial preparation of stable unlabeled DNA, that can be conveniently stored until the radioactive probe is desired.

We have conducted a number of studies using viral DNA probes labeled in vitro. We have used Ad DNA ($2-4 \times 10^8$ cpm/μg) to (i) precisely quantitate the number of copies and the fraction of the viral genome persisting in Ad12 transformed cells and Ad12 induced tumors (Green et al., 1976a, 1977), (ii) detect nuclear viral RNA species present in very low abundance and not transported to the cytoplasm of Ad transform cells (Green et al., 1976b), and (iii) exclude the presence of 1-2% of the Ad12 genome in human neoplasms (Mackey et al., 1976). In vitro Ad12 DNA probes are sensitive enough to detect as little as 0.2 copy per diploid cell of a fraction of the Ad12 genome and have provided the first definitive negative answers regarding an Ad12 etiology of human GI and lung cancer. These experiments could not have been performed at this level of sensitivity with in vivo probes with specific activities of 10^7 cpm/μg or less.

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Intermediates in Excision Repair by Human Cells: Use of S_1 Nuclease and Benzoylated Naphthoylated Cellulose to Reveal Single-Strand Breaks†

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ABSTRACT: Treatment of human lymphoid cells with methyl methanesulfonate (MMS) results in discontinuities in cellular DNA. Discontinuities can be detected by sedimentation through neutral sucrose gradients after digestion with S_1 nuclease on top of the gradient and by benzoylated naphthoylated diethylaminoethylcellulose (BND-cellulose) chromatography. DNA with discontinuities is sensitive to shear which produces single-stranded regions and causes the DNA to adhere to BND-cellulose. The shear-sensitive and S_1-sensitive sites produced by MMS treatment are probably identical. Treatment of cells with acetoxyacetylaminofluorene (AAAF) does not result in detectable discontinuities either at doses which produce the same number of adducts as observed after MMS treatment or at doses which inhibit DNA excision repair. Addition of aromatic adducts to DNA can also result in adherence to BND-cellulose but such DNA is not S_1 sensitive. We conclude that MMS- and AAAF-induced damage are repaired by different pathways in human cells and that DNA with discontinuities is a readily detectable intermediate only in the repair of MMS damage by the apurinic pathway.

There are at least two modes of DNA excision repair in human cells. "Long patch" repair is the typical response to ultraviolet-light-induced damage and results in repair segments of the order of 100 nucleotides (Regan and Setlow, 1974). "Small patch" repair is induced by treatment of human cells with ionizing radiation and at low doses results in patch sizes of 3–4 nucleotides (Painter and Young, 1972; Regan and Setlow, 1974).[1] A variety of chemical agents combine with DNA

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[1] "Long patch repair" has been given a different meaning for bacteria and refers, in such organisms, to an excision repair process which introduces patches of about 1000 nucleotides and requires the *rec* gene products as well as protein synthesis (Cooper and Hanawalt, 1972). This paper deals with excision repair in the sense defined for human cells by Regan and Setlow (1974).