DNA Chain Termination by 2′,3′-Dideoxythymidine in Replicating Mammalian Cells†

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ABSTRACT: The thymidine analog, 2′,3′-dideoxythymidine (ddT), is rapidly phosphorylated and incorporated terminally at 3′-ends of growing DNA chains in replicating mammalian cells. Following some initial loss of ddT incorporated into DNA chains, the major portion is retained for periods equivalent to more than two normal cell generations. Some ddT appears at the termini of oligonucleotides, a portion of which have chromatographic properties suggesting internally complementary sequences. While these oligonucleotides may include degradation fragments, it is possible that some represent replication initiation sequences.

Useful information concerning both mechanisms and initiation signals for mammalian DNA replication might be gained if it were feasible to attack systematically the problem of base sequence analysis of initiation regions. One way in which a rational approach could be made is to terminate newly initiated daughter strands at points which are a relatively short distance from the initiation positions. In theory, this might conceivably be achieved by using pulsed application of chain-terminating nucleosides.

The thymidine triphosphate analog 2′,3′-deoxythymidine triphosphate can be used as a substrate by DNA polymerase I of *Escherichia coli* and incorporated into growing DNA chains in vitro, whereupon the chains terminate because of the lack of the 3′-hydroxyl group necessary for chain continuation (Atkinson et al., 1969). The nucleoside analog 2′,3′-dideoxyadenosine irreversibly inhibits DNA synthesis in *E. coli* (Doering et al., 1966). In the present study an attempt has been made to utilize the chain-terminating function of 2′,3′-dideoxythymidine (ddT)[1] in mammalian cells to ascertain its possible value for analysis of replication in complex systems. In contrast to the situation in some bacteria, this nucleoside analog is phosphorylated and incorporated into DNA very rapidly in the mammalian cell lines tested. This makes it possible to do kinetic experiments over short time periods and to use chain termination as a means of isolating short DNA fragments.

Materials and Methods

Materials. 2′,2′-Dideoxythymidine, 2′,3′-dideoxythymidine 5′-phosphate, and [^{3}H]-2′,3′-dideoxythymidine (35 Ci/mmol) (cf. Pfitzner and Moffatt, 1964) were kindly supplied by Dr. J. G. Moffatt. [^{3}H]Thymidine (30 Ci/mmol) and [^{14}C]thymidine (50 Ci/mol) were obtained from New England Nuclear Corp. Exonuclease I was a gift from Dr. I. R. Lehman. Akaline phosphatase, calf spleen phosphodiesterase, micrococcal nuclease, venom diesterase and pancreatic deoxyribonuclease I (RNase-free) were obtained from Worthington Corp. Oligo(T) markers (Khorana and Vizsolyi, 1961) were generously provided by Dr. J. G. Moffatt. $(Ap)_5A$ was obtained from Miles Laboratories.

Cell Culture. Cl and XCl lines of the mouse myeloma X5563 were grown in suspension culture as described previously (Byars and Kidson, 1970). Mouse L929 cells and Chang liver cells were grown as monolayers in Eagle's minimal essential medium (Gibco). Where required, cell growth was synchrony using a double thymidine block and criteria of synchronizing described previously (Byars and Kidson, 1970), except that for L929 cells the thymidine concentration used was 2 m*M*.

DNA Isolation. To prepare labeled DNA, cells were incubated with 10 μCi/ml of [^{3}H]-2′,3′-dideoxythymidine for 1 hr at 37°. When synchronized cell cultures were employed, labeling was carried out in designated portions of S phase, usually during the second hour. After labeling, cells were harvested, washed, and DNA extracted by the phenol method (Kidson, 1966), except that triisopropylnaphthalenesulfonate was used as detergent. Salt and traces of phenol were removed by gel filtration on Sephadex G-25 (Pharmacia), to prevent loss of small oligonucleotides. Prior to filtration the gel column was equilibrated with 10 m*M* triethylammonium bicarbonate (pH 7.8) in preparation for the following step.

Ion-Exchange Chromatography. Chromatography on Sephadex A-25 (Pharmacia) was used to prepare and analyze small oligonucleotides found with the bulk DNA (Khorana and Vizsolyi, 1961). After loading, 20-ml columns were washed with 85 ml of 10 m*M* triethylammonium bicarbonate, then oligonucleotides were eluted with a linear gradient of 10 m*M* to 1 *M* triethylammonium bicarbonate. Since in the initial runs it was found that most of the oligonucleotides treated with alkali during isolation were eluted well below 1 *M* salt, in some subsequent runs gradients of 10–600 m*M* were used. Fractions were bulked as indicated and concentrated by lyophilization, a step which also served to remove the triethylammonium bicarbonate.

Where indicated, DNA was denatured prior to Sephadex A-25 chromatography, by adding NaOH to pH 12.8 for 60 min, followed by titration with Dowex 50 (Bio-Rad Laboratories) to pH 7.8.

Hydroxylapatite Chromatography. The presence of single- or double-stranded DNA and oligonucleotides was determined by chromatography on hydroxylapatite (Bio-Rad Laboratories). Both stepwise (0.01, 0.1, 0.18, and 0.4 *M*

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[1] Abbreviations used are: ddT, 2′,3′-dideoxythymidine; ddTMP, 2′,3′-dideoxythymidine 5′-monophosphate; ddTDP, 2′,3′-dideoxythymidine 5′-diphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate.

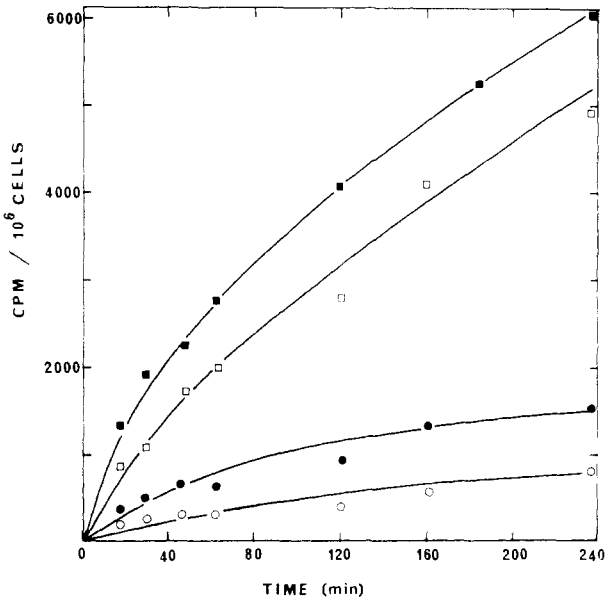

FIGURE 1: Incorporation of [^{3}H]ddT (2.6×10^{-7} *M*; 10 μCi/ml) into DNA as a function of time in Cl (□), XCl (■), L929 (●), and Chang liver (○) cells in exponential culture.

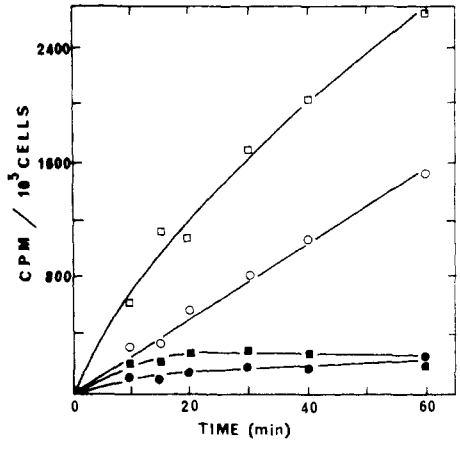

FIGURE 2: Uptake of [^{3}H]thymidine (2×10^{-7} *M*; 1 μCi/ml) into DNA of XCl (□) and L929 (○) cells and its inhibition by 1 m*M* ddT (XCl, ■; L929, ●) as a function of time.

phosphate (pH 6.8)) and gradient (0.01–0.6 *M* phosphate) elution systems were used. The columns were standardized by cochromatographing [^{3}H]thymidine-labeled single-stranded DNA and [^{14}C]thymidine-labeled double-stranded DNA: these were eluted respectively at 0.15 and 0.28 *M* phosphate.

Characterization of Oligonucleotides. Exonuclease I digestion was carried out as described by Lehman (1960). The reaction was stopped either by precipitation with trichloroacetic acid or by chilling and immediately loading the incubation mixture on to a 1 × 21 cm Bio-Gel P2 (Bio-Rad Laboratories) column.

Digestion of extensively dialyzed [^{3}H]ddT-labeled DNA with pancreatic DNase I was performed in 0.1 *M* sodium acetate–25 m*M* $MgCl_2$ (pH 7) for 2 hr at 37°. The digest was then dialyzed extensively against 0.5 *M* NaCl–0.1 *M* sodium acetate. Trichloroacetic acid was added to the contents of the dialysis sack to give a final concentration of 5%, the precipitate was collected on a Millipore filter (0.45 μ), and radioactivity was determined by liquid scintillation spectrometry using a toluene phosphor.

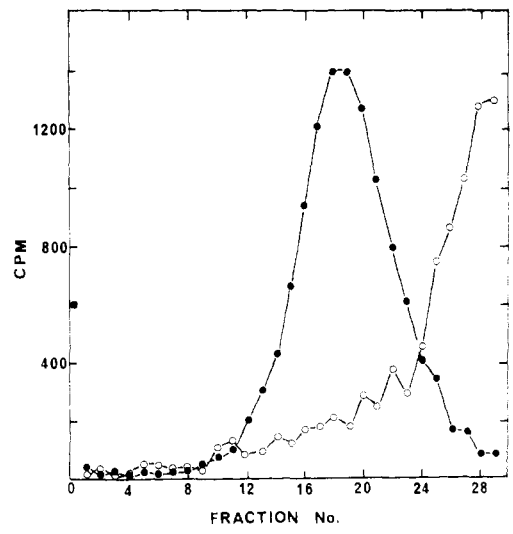

FIGURE 3: Alkaline sucrose gradient analysis of DNA from XCl cells labeled for two generations with [^{14}C]thymidine (0.1 μCi/ml) and pulse-labeled for 1 hr with [^{3}H]ddT (10 μCi/ml). Material applied to the gradient represents the exclusion peak from a Sephadex G-25 column. Samples were centrifuged for 1 hr at 4° at 50,000 rpm in the SW56 rotor of a Spinco L2-65B ultracentrifuge. Fractions were collected on GF/C filters, dried, and counted. Radioactivity at the top of the gradient includes some mononucleotides of ddT. [^{14}C]Thymidine (●), [^{3}H]ddT (○).

Micrococcal nuclease and spleen diesterase digestions were carried out by the method of Josse et al. (1961) and venom diesterase digestion was carried out by the method of Lehman et al. (1958). Products were separated and identified by descending paper chromatography in the isobutyrate system (isobutyric acid–2 *M* NH_4OH–0.2 *M* Na_2EDTA, 120:70:2), with the aid of appropriate marker compounds.

Incubation of oligonucleotides with alkaline phosphatase was carried out in 10 m*M* Tris (pH 8.0) for 45 min at 37°. After incubation, samples were frozen, lyophilized, and prepared for paper chromatography, paper electrophoresis, or acrylamide gel electrophoresis.

Acrylamide gel electrophoresis was carried out as follows: 10% gels prepared in 8 *M* urea, 0.4 *M* Tris, 0.02 *M* sodium acetate, and 2 m*M* Na_2EDTA (pH 7.6) were run for 1 hr at 5 mA/tube. Gels were cut into 1- or 1.5-mm slices, heated in 0.7 ml of 10% piperidine at 56° until dry, rehydrated in 0.5 ml of water, and radioactivity assayed by liquid scintillation spectrometry using a dioxane phosphor. The marker $(Ap)_5A$ was visualized by staining with 0.2% Methylene Blue. When $(Tp)_6$ and $(Tp)_{10}$ were used as markers, 0.01% Radelin phosphor was incorporated during preparation of the gels and the markers visualized under uv light.

Cell Incorporation Experiments. Cell cultures were incubated with [^{3}H]ddT (10 μCi/ml medium) for various times up to 4 hr. The cells were harvested on Millipore filters (0.45 μ) and washed with cold isotonic saline and followed by 5% trichloroacetic acid. The filters were dried and radioactivity assayed by liquid scintillation spectrometry in a toluene phosphor. To determine if incorporated [^{3}H]ddT nucleotides were excised with time, synchronized cell cultures were incubated for 1 hr with the labeled nucleoside, then washed and allowed to grow in fresh medium without labeled nucleoside for up to 48 hr longer. In some experiments trichloroacetic acid precipitable radioactivity was measured; in others DNA was extracted as described above

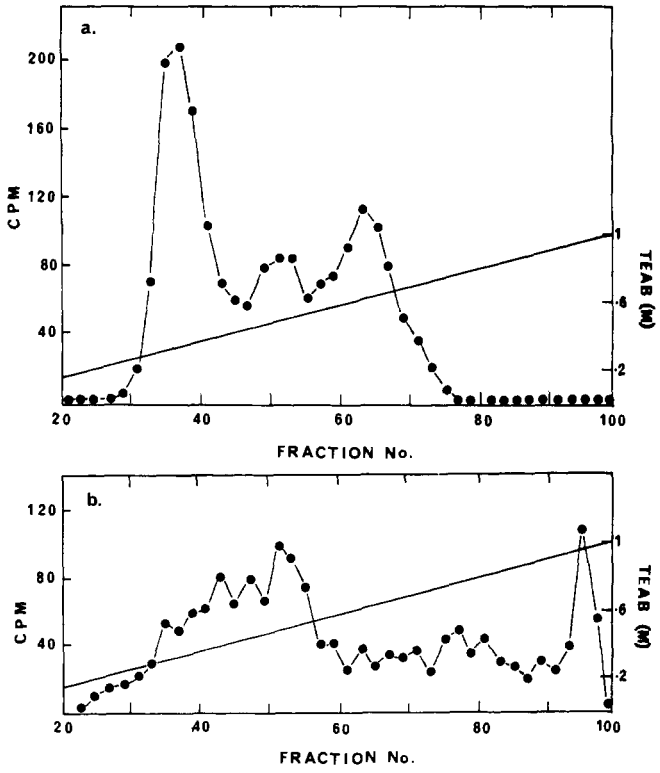

FIGURE 4: Chromatography on Sephadex A-25 of [^{3}H]ddT-labeled (2.6×10^{-7} *M*, 10 μCi/ml for 1 hr) material from Sephadex G-25. Elution with triethylammonium bicarbonate containing 8 *M* urea. Aliquots from fractions counted in a dioxane phosphor. (a) Preparation denatured by alkali and titrated to pH 7.8 with Dowex 50; (b) preparation not denatured.

and the specific radioactivity determined as a function of time.

To measure the inhibition of thymidine incorporation by ddT, cell cultures were incubated at 37° for various times with 1 m*M* ddT and [^{3}H]thymidine. This level of ddT was chosen following initial experiments which examined the effects of a concentration range of the nucleoside from 10 n*M* to 1 m*M*. The cells were harvested on Millipore filters, washed with cold, isotonic saline, followed by 5% trichloroacetic acid, then the filters were dried and counted as above.

Results

Incorporation of [^{3}H]-2′,3′-Dideoxythymidine. The rates of incorporation of the labeled nucleoside into trichloroacetic acid precipitable material in four different cell lines are shown in Figure 1. These rates varied with the cell line, but all showed initial rapid rates followed by slower, linear incorporation rates over several hours. Careful analysis of initial rates showed that demonstrable nucleoside incorporation occurred in less than 1 min. Paper chromatography of acid-soluble material showed the presence of intracellular ddT, ddTMP, ddTDP, and ddTTP at short time periods.

DNA labeled with [^{3}H]ddT, isolated by the phenol method, then digested for 2 hr with DNase I was largely (>75%) converted to dialyzable products. Together these data indicate that 2′,3′-dideoxythymidine is phosphorylated and incorporated into DNA very rapidly in these cell lines. The murine myelomas, Cl and XCl, appeared consistently to incorporate more labeled nucleoside per cell in a given time than did the other cell lines; XCl cells were used in most subsequent experiments.

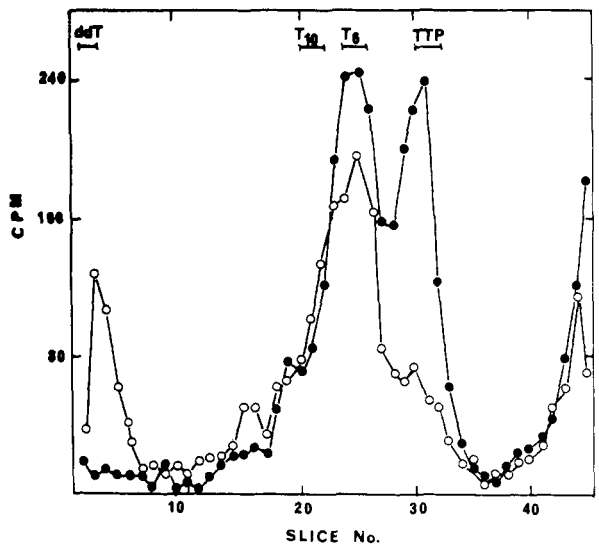

FIGURE 5: Electrophoresis on polyacrylamide gels of denatured material recovered from Sephadex A-25. (●) Before alkaline phosphatase, (○) after alkaline phosphatase. Marker positions as shown.

Inhibition of [^{3}H]Thymidine Incorporation. The incorporation of [^{3}H]thymidine into XCl DNA was found to be inhibited by ddT, inhibition being greater than 90% at an inhibitor concentration of 1 m*M*. Inhibition was rapid, was maintained over the time course studied (Figure 2), and was similarly observed in L929 cells.

Sucrose Gradient Analysis. A rough sizing of the 2′,3′-dideoxythymidine labeled polynucleotides was achieved by sedimentation in alkaline sucrose density gradients. XCl DNA parental strands were labeled over two generations with [^{14}C]thymidine; the cells were washed with warm Hank's solution, resuspended in prewarmed growth medium, and DNA daughter strands pulse-labeled with [^{3}H]ddT for 1 hr. DNA isolated by the phenol method was taken from Sephadex G-25 and sedimented in a preformed sucrose gradient (Figure 3). The pulse-labeled material sedimented in a heterogeneous pattern, the great majority being considerably smaller than the parental DNA strands. [^{3}H]Thymidine-labeled parental DNA sedimented in the same position as [^{14}C]thymidine-labeled parental DNA in a control experiment.

[^{3}H]-2′,3′-Dideoxythymidine-Labeled Oligonucleotides. The chromatography on Sephadex A-25 of ^{3}H-labeled material from Sephadex G-25 resulted in elution of radiolabeled material between 0.2 and 0.6 *M* triethylammonium bicarbonate (denatured DNA preparation in the presence or absence of 8 *M* urea, Figure 4a) or between 0.2 and 1.0 *M* triethylammonium bicarbonate (native DNA preparation, Figure 4b). Multiple peaks were seen in each case. The elution position suggests that some, at least, of the labeled material consists of oligonucleotides.

Electrophoresis on polyacrylamide gels (Figure 5) of denatured material recovered from Sephadex A-25 showed that some of the label was sensitive to alkaline phosphatase, being converted to 2′,3′-dideoxythymidine and evidently represents nucleotides derived from this nucleoside. A large proportion of the radiolabeled material was insensitive to alkaline phosphatase and would appear, from comparison with marker oligomers, to represent oligonucleotides of varying chain lengths. The data from Sephadex A-25 chro-

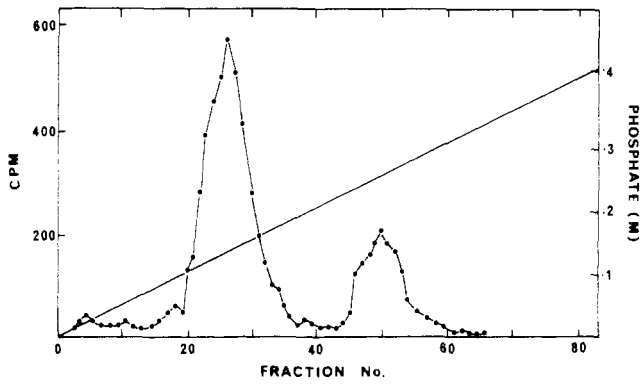

FIGURE 6: Chromatography on hydroxylapatite of preparatively denatured oligonucleotides from Sephadex A-25.

matography in 8 *M* urea and those from polyacrylamide electrophoresis are consistent with chain lengths in the range 2–20, but such estimates are very approximate at best, in the absence of any base composition or sequence data. The majority of oligonucleotides appeared to be fairly short.

Quantitative estimation of the proportion of [^{3}H]ddT incorporated into these oligonucleotides as against polynucleotides was difficult owing to the amount of contaminating mononucleotides. However, approximations suggested that this proportion may be as high as 10–15% of the total. This is interesting in view of the fact that the concentrations of the radiolabeled nucleoside used (2.6×10^{-7} *M*) were several orders of magnitude below that required to produce complete or nearly complete inhibition of [^{3}H]thymidine incorporation into DNA.

Enzyme Analysis of Oligonucleotides and DNA. DNA and oligonucleotide fractions recovered from Sephadex A-25, in each case containing [^{3}H]ddT, were analyzed by the use of standard enzymatic methods to determine the position of the radiolabel. Digestion with micrococcal nuclease and spleen diesterase gave rise to labeled ddT and some residual labeled oligomers, probably of the type XpddT and XpYpddT. Digestion with venom diesterase gave rise to labeled ddTMP and some residual labeled oligomers, but no free nucleoside. The [^{3}H]-2′,3′-dideoxythymidine was thus incorporated at the 3′-ends of the oligonucleotides and of the DNA chains.

Digestion by exonuclease I of denatured DNA labeled with [^{3}H]ddT showed that chains terminated with this nucleoside were relatively resistant to the enzyme, compared with [^{3}H]thymidine-labeled DNA. These findings suggest that exonuclease I is unable to hydrolyze chains where the terminus contains a 3′-H rather than a 3′-OH. The enzyme is known to be blocked by the presence of a phosphate group at the 3′ position (Lehman, 1960).

Hydroxylapatite Chromatography of Oligonucleotides. Oligonucleotides recovered from Sephadex A-25 and then chromatographed on hydroxylapatite showed unexpected properties. After washing contaminating mononucleotides from the columns below 0.1 *M* phosphate, oligonucleotides were eluted in two peaks, the first at about 0.15 *M* and the second at about 0.25 *M* phosphate (Figure 6). These elution positions corresponded roughly with those for denatured DNA and native DNA, respectively. In the absence of sequence data and hence of suitable model compounds, it is not known if the behavior of these oligonucleotides on hydroxylapatite chromatography should be expected to parallel that of the larger DNA polymers, but the possibility cannot be excluded that some of the oligonucleotides possess a degree of secondary structure. This chromatographic behavior was observed with oligonucleotides prepared with or without prior alkali denaturation of the DNA preparation. If secondary structure is the basis for the chromatographic behavior of the oligonucleotide fraction eluted at the higher phosphate concentration, it must thus be capable of reforming following neutralization after alkali denaturation.

Table I: Persistance of ddT-Labeled DNA over Several Cell Generations.[a]

Mitosis	Time (hr)	Cpm/10^5 Cells	DNA (Relative to Zero Time = *x*)
	2	550	1.3*x*
	4	340	1.7*x*
	6	350	2*x*
M1→			
	24	220	4*x*
	28	210	4*x*
M2→			
	48	105	8*x*

[a]Cells pulse labeled with [^{3}H]ddT for 1 hr between hours 1 and 2 of S phase. Trichloroacetic acid precipitable radioactivity assayed at stated times in the initial generation and at the end of S phase in two subsequent cell generations.

Long-Term 2′,3′-Dideoxythymidine Incorporation. Synchronized XCl cultures were pulse-labeled with [^{3}H]ddT early in S phase, washed with warmed Hank's solution to remove residual labeled nucleoside, resuspended in prewarmed growth medium, and allowed to continue growth for up to 48 hr. Acid-precipitable radioactivity was present throughout this period (Table I). There was an initial fall in specific radioactivity immediately following the labeling period. Subsequently the specific radioactivity remained essentially constant, allowing for DNA duplication, computed from cell numbers. This implies that much of the nucleoside incorporated initially into longer DNA chains persists through a period equivalent to more than two normal cell generations. In experiments in which XCl cells were pulse-labeled early in S phase and DNA plus oligonucleotides isolated as above at various times during this first S phase, labeled oligonucleotides were no longer present late in S phase, in contrast to the larger labeled DNA chains.

Cell Growth. Cells synchronized by double thymidine block were pulsed for varying times (10, 30, or 60 min) with 1 m*M* ddT, starting immediately after release of the second thymidine block. Cells were then washed and resuspended in growth medium and growth curves followed over the subsequent 50–70 hr. Average growth curves compared with controls (treated identically but without ddT) are shown in Figure 7. At longer pulse times there was a measurable lag in growth rate but eventually growth rates were largely similar to those of the controls. This suggests either that only a proportion of the population survive, especially at longer pulse-times, or that time is required for repair or for adaptive utilization of alternative metabolic pathways to circumvent those dependent on damaged regions of the genome. If only a proportion of the population survives it is of course possible that this represents cells which have taken up little or no ddT.

Discussion

Although 2′,3′-dideoxythymidine triphosphate can be

used as a substrate by *E. coli* DNA polymerase I and thus incorporated into DNA, the corresponding nucleoside is a very poor substrate for *E. coli* thymidine kinase (Atkinson et al., 1969). In contrast, in the mammalian cells tested here, 2′,3′-dideoxythymidine is readily phosphorylated and is incorporated into DNA as a chain terminator at 3′-ends. These observations, together with the rapid rate of incorporation into DNA of this analog and its ability to compete with thymidine in this respect, suggest it is a useful probe of mammalian DNA replication and repair.

Evidence from alkaline sucrose density gradient centrifugation (Figure 3) indicates that there was a spectrum of short, labeled DNA strands, as would be expected from random incorporation of ddTMP at ends of growing daughter chains. Experiments using synchronized cell populations, in which the fate of an early S phase pulse of [^{3}H]ddT was followed for a period equivalent to two normal cell generations, indicated that during this time, there was some initial loss of incorporated labeled nucleoside, but a substantial proportion was retained in longer DNA chains during that period. This observation implies that some excision and possibly repair occur early but that many of the terminated chains are retained, without repair involving excision of the blocked termini. The data say nothing, of course, about the unlikely possibility that complete resynthesis of these terminated chains might occur, with the latter being discarded in the functional sense but not degraded during the time studied, and so continuing to be monitored. Absence of the 3′-hydroxyl may render them relatively resistant to exonuclease attack, for example.

Of considerable interest is the isolation of oligonucleotides terminated at their 3′-ends with ddT. The use of ion-exchange chromatography for separation of these oligonucleotides gives an arbitrary cut-off point on the basis of size and necessarily means that no upper limit can be given. On this rough basis, nevertheless, the majority appeared to be small and few if any oligonucleotides were eluted in the range 0.6–1.0 *M* salt, when the preparation was denatured prior to chromatography.

Some of these oligonucleotides may represent degradation fragments resulting from repair of aborted chains terminated prematurely by ddT, as noted above. Generation of degradation fragments in this manner would have to occur fairly early after premature termination of daughter strands since oligonucleotides labeled early in S phase were no longer identified in DNA preparations isolated late in S phase of synchronized cell populations. Some fragments terminating in ddT would be expected to occur as the result of aborted daughter strand synthesis and as such should represent initiation deoxyribonucleotide sequences. This would be true if (a) they were attached in vivo in a polyribonucleotide primer (cf. Sugino et al., 1972) which was removed by alkali during the isolation procedure, or if (b) they were free from attachment to a parental DNA strand (cf. Haskell and Davern, 1969) or other polydeoxyribonucleotide primer, or to another daughter strand segment. With respect to (a), it is noteworthy that preparations isolated without a denaturation step showed a continuum of labeled oligonucleotides on elution with up to 1.0 *M* salt (Figure 4b): oligodeoxyribonucleotides attached to polyribonucleotide primers, by virtue of their chain length, would be expected to be difficult or impossible to elute from DEAE-cellulose under these conditions; certainly elution would only be expected at high salt concentrations. With respect to (b), detachment might occur where a fragment was too short be become

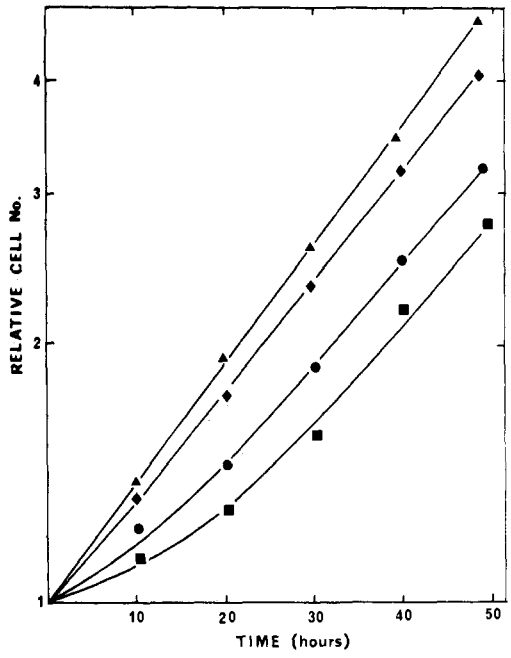

FIGURE 7: XCl growth rates after pulse application of 1 m*M* ddT for 10 min (♦), 30 min (●), and 60 min (■) compared with controls (▲).

joined at its 5′-end to the preceding, completed daughter strand segment by polynucleotide ligase.

It is quite conceivable that termination very soon after chain initiation, before the oligodeoxyribonucleotides are sufficiently long to form stable duplexes with the corresponding regions of parental strands, may lead to a stuttering effect, whereby a series of short fragments, each terminated in ddT, is generated on the same region of a parental template strand. This could possibly account, in part at least, for the rather higher than expected proportion of oligonucleotides compared with polynucleotides terminated with ddT. In this context, however, there still remains the problem of this high proportion of oligomers vis à vis the low ddT concentration relative to that required to inhibit the incorporation of thymidine. This might be largely explained by the presence of degradation fragments. However, the level of competition ddT with thymidine is unknown: ddT may compete poorly with thymidine at other levels; e.g., cell entry or phosphorylation. Another possibility is that at least some replication initiation sequences contain a high proportion of thymidine residues, thus increasing the chances of incorporation of an analog of TMP.

Interpretation of the chromatographic behavior of the oligonucleotides on hydroxylapatite is not clear. If the second peak does indeed reflect the existence of a degree of secondary structure, this implies the presence of sequences which are internally complementary, since this behavior occurred following alkali denaturation and subsequent neutralization. If such sequences occur near the beginnings of some daughter strands this would indeed be interesting and possibly of signal significance. Further interpretation must await sequence data.

In addition to using ddT as a probe for analysis of DNA replication and repair, the finding that longer terminated DNA molecules persist in growing cell populations raises the possibility of using this nucleoside analog for introducing mutations. Studies with diploid and aneuploid human cell lines indicate that this compound is indeed a good mutagen (Kidson et al., 1975).

Acknowledgments

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A Comparison of the Binding to Polynucleotides of Complementary and Noncomplementary Oligonucleotides†

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ABSTRACT: The binding of oligonucleotides to synthetic polynucleotides has been studied as a control for investigations of the binding of oligonucleotides to natural RNA molecules. Only combinations that involved A-U, G-C, and G-G pairs were found to be significantly stable under the experimental conditions used here. The stability of the oligomer–polymer pairing increased with the length of the region paired and with its G + C content. Further, some different sequence isomers of the same G + C content exhibited quite different binding constants. This variability is consistent with certain sequence differences in the double-strand stacking interactions stabilizing the oligomer–polymer association. Oligomer binding was also shown to depend upon the identity of the polymer residues neighboring the binding site, indicating the effect upon oligomer binding of small changes in the single-strand conformation of the binding site. These observations validate the criteria that allow one to decide if an observed association constant of an oligomer to an RNA molecule reflects a complete complementarity between the two or not. This improves the basis for using oligonucleotide binding constants to RNA of known sequence to map secondary structure.

Recent reports (Uhlenbeck et al., 1970; Lewis and Doty, 1970; Hogenauer, 1970; Danchin and Grunberg-Manago, 1970; Uhlenbeck, 1972; Schimmel et al., 1972) have used the binding of short oligonucleotides to RNA to investigate the secondary structure of small RNAs of known sequence. The supposition is that only those parts of the RNA sequence that are single stranded and available for base pairing will bind complementary oligonucleotides. Thus the sequence of the regions of the RNA chain that are single-stranded and available can be deduced from the sequences of the oligonucleotides that are found to bind to the RNA by assuming that the binding site for the oligonucleotide on the RNA molecule is the Watson–Crick antisequence of the oligomer. For this assumption to be valid requires first that only Watson–Crick specific interactions contribute significantly to the negative free energy of binding. Second, it is necessary that the binding constant for any part of an oligonucleotide sequence be sufficiently less than the binding constant for the entire sequence that the single-stranded region of the RNA can be identified as the complement of the entire oligonucleotide.

To determine if these two conditions are met, we have studied the binding of oligonucleotides to synthetic polynucleotides. The frequency with which various site sequences occur can be calculated on the basis of the random incorporation of the nucleotides into the polymer (Grunberg-Manago, 1963). The effect of secondary structure masking some of these sites, as is expected to occur with natural RNAs, should be eliminated by using only those bases in a given polymer that will not pair to each other. It will be seen, however, that in a few cases secondary structure still persists, and this structure must be considered in interpreting experiments with these few polymers. Each of the effects mentioned above can be studied by systematically varying the sequence of the oligomers used and the composition of the polymer.

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