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Antiviral Effect of Phosphorothioate Oligodeoxyribonucleotides Complementary to Human Immunodeficiency Virus

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Abstract—Modifications of oligodeoxyribonucleotides include the replacement of the backbone phosphodiester groups with phosphorothioate (S-ODNs) groups and the substitution of phosphorothioate (SO-ODNs) groups at both the 3'- and 5'-ends. In assays for HIV, oligomers (S-ODNs) were more active at the micromolar range than were SO-ODNs of the same sequence. Furthermore, the abilities of antisense-, sense-, random-, and mismatched-oligomers, or homo-oligomers containing internucleotidic phosphorothioate linkages to inhibit HIV-1 replication were examined. Antisense oligonucleotides inhibit the replication and the expression of HIV-1 more efficiently than random-, sense-, mismatched-, and homo-oligomers of the same length or with the same internucleotide modification. Five different target sites (*gag*, *pol*, *rev*, *tat*, and *tar*) within the HIV genes were also studied with regard to the inhibition of HIV replication by antisense oligonucleotides. Antisense oligomers complementary to the sites of initiation sequences and to certain splice sites were most effective. The effect of antisense oligomer length on inhibiting viral replication was also investigated. Of particular interest was the S-ODNs-*rev* 15 mer, which possessed higher anti-HIV activity than the sense-, random-, mismatched-, and homo-20 mers.

Introduction

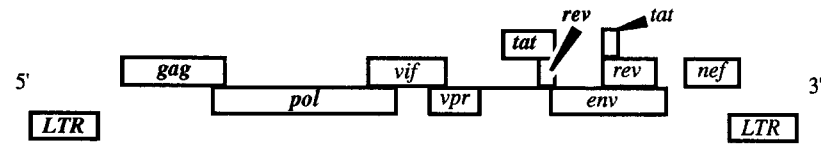
Antisense oligonucleotides and their analogues have been shown to inhibit viral replication,[1–3] to block the splicing and translation of mRNA,[4,5] and to regulate specific gene expression.[6,7] An oligonucleotide complementary to a viral genome or to its mRNA may interfere with the expression of that genetic segment by hybridization.[1,2]

The stability of the oligonucleotide in the culture medium and within the cell will influence its inhibitory effect. It has been demonstrated that unmodified oligonucleotides have limited use *in vitro* as well as *in vivo*, because of their sensitivity to cellular nucleases, which are present in the culture medium and serum.[8–10] To overcome this problem, the backbone phosphates of some antisense oligonucleotides have been modified to methylphosphonates[11,12] or phosphorothioates (S-ODNs),[9] and S-ODNs have proven anti-HIV activity in acute infections *in vitro*.[9, 13–16] Matsukura *et al.* reported that the inhibition of *de novo* infection by S-ODNs is both composition- and length-dependent, for example, homo-oligo S-dC_{28} is a better inhibitor than either S-dC_{20} or S-ODNs (20mer, encoding exon I of the *art/trs* gene in HIV-1).[9] However, in chronically infected T cells, S-dC_{28} did not inhibit p24 *gag* expression, whereas S-ODNs complementary to the initiation sequence of HIV-*rev* inhibited the production of several viral proteins.[17] On the other hand, Lisziewicz *et al.*[18] have reported that chemotherapy based on specifically targeted antisense oligonucleotide phosphorothioates is an effective means of reducing the viral burden in HIV-1 infected individuals, at clinically achievable oligonucleotide concentrations. In a previous paper, we reported the inhibition of HIV-1 replication in acutely HIV-1 infected cells by phosphorothioate oligonucleotides.[10] To define the dependence upon both the target sequences and the length of the phosphorothioate oligonucleotides for optimal anti-HIV activity, we synthesized phosphorothioate oligonucleotide analogues. In this paper, we extend those observations with a comparison of oligonucleotides with either backbone of phosphorothioate groups or those capped with a phosphorothioate group at both the 3' and 5'-ends, in sense, random, mismatched, homo-oligomeric, or antisense sequences, with five different target sites (*gag*, *pol*, *rev*, *tat*, and *tar*) within the HIV-1 gene (Fig. 1).

Results and Discussion

Sequence-specific inhibition of viral expression in HIV-1 infected cells

To examine the selective antisense inhibition of HIV with a phosphorothioate oligomer, we selected targets



GGUCUCUCUGGUUAGACCAG(1-20)
3' d(CCA GAG AGACCAATCTGGTC) S-ODNs-TAR-1

Tat protein binding

GACCAGAUCUGAGCCUGGGA(15-34)
3' d(CTGGT CTAGACTCGGACCCT) S-ODNs-TAR-2

CUGGCUAACUAGGGAACCCA(40-59)
3' d(GACCGATT GATCCC TTGGGT) S-ODNs-TAR-3

splice acceptor

CAAAAGCCUUAGGCAUCUCC(5529-5548)
d(CCTCTA CGGATTCCGAAAAC) S-ODNs-rev-sa-sen
d(GTTTTC GGAATCCGTAGAGG) S-ODNs-rev-sa-anti
d(GTTTTCGGAATCAGTAGAGG) S-ODNs-rev-sa-8mis
3' d(GTTTCCGGAATCCGTAGAGG) S-ODNs-rev-sa-16mis
d(AGTACGACTGGGTGTAGTCT) S-ODNs-ran
d(CGGAATCCGTAGAGG) S-ODNs-rev-sa-15
d(TCCGTAGAGG) S-ODNs-rev-sa-10

p24 leader

CCUAUAGUGCAGAACAUCCA(1185-1214)
d(GGATATCACGTCTTGTAGGT) S-ODNs-gag-ls 5'

splice acceptor

UUAUUACAGGGACAGCAGAG(4486-4505)
d(AAT AAT GTCCCTGTCGTCTC) S-ODNs-pol-sa 5'

splice acceptor

CCAUUUUCAGAAUUGGGUGU(5348-5367)
d(GGTAAAAGT CTT AACCCACA) S-ODNs-tat-sa 5'
*d(GGTAAAAGT CTT AACCCACA) LS-ODNs-tat-sa

translational start

AUGGCAGGAAGAAGCGGAGA(5550-5569)
d(TAC CGT CCT TCT TCGCCTCT) ODNs-rev-ts
d(TAC CGT CCT TCT TCGCCTCT) S-ODNs-rev-ts 5'

Figure 1. DNA sequences of the leader site of *gag*, the splice acceptor sites of *pol*, *rev*, and *tat*, and the translation start site of *rev*. The random sequence has the same base content as antisense *rev*-sa , but has < 70% homology with any portion of the HIV sequence, as either antisense or sense. Homo-oligomers of the four bases (A, C, G, and T) were synthesized in four lengths (10, 15, 20, and 28 mer). The phosphorothioate derivatives are denoted by 'S'.

that were involved in the viral recognition sites (Fig. 1). These include the extreme 5'-end of the RNA, and all of the AUG initiator codons and the splice acceptor sites involved in the processing of the RNA. The *pol* gene does not have an initiator AUG, but instead starts by an occasional frame shift of ribosomes translating the *gag* mRNAs. Splice sites are good targets for HIV,[16] and also for herpes simplex virus.[3] These sites, which are involved in the translation and processing of RNA, are expected to have some homology with host sequences. Therefore, long oligonucleotides should bind the adjacent viral sequences and thus may be more specific.

All the antisense oligomers inhibited HIV-1 replication without toxicity in acutely HIV-1 infected cells (Table 1) and, moreover, the antisense S-ODNs of the targeted internal splice sites (*rev* and *tat* splice acceptor sites) and the initiator codon (AUG) were very active, causing more than 95% inhibition at 0.02–0.5 μM. The S-ODNs-*pol*-sa, however, failed to satisfactorily prevent HIV-1 replication (up to 64% inhibition at 0.02 μM). On the other hand, the S-ODNs-*gag*-ls contains a sequence complementary to the leader sequence of p24 (CCU), which may play an important role in mRNA processing, and its activity (> 92% inhibition at 0.02–0.5 μM) was as good as those of the internal, valid splice sites or the initiator codon (Table 1).

On the other hand, we could not detect any inhibitory effects of the 3',5'-capped, phosphorothioate-substituted oligomer (SO-ODNs-*rev*-sa) at a concentration of 0.5 μM (Table 1). The weaker anti-HIV activity of the SO-ODNs oligomer than that of the S-ODNs oligomer was supported by the uptake studies.[19–21] Figure 2 shows the effect of time on the uptake of the 3'-[^{32}P] labeled SO-ODNs-*tat*-sa and S-ODNs-*tat*-sa by MOLT-4 cells. The uptake reached a plateau after 60 min, and the SO-ODNs oligomer yielded about 10 times less cell-associated counts than the S-ODNs oligomer. This 3',5'-capped phosphorothioate group (SO-ODNs) was weakly assimilated by MOLT-4 cells in cooperation with the phosphorothioate oligomers (S-ODNs), and decreased the antiviral efficiency of the phosphorothioate oligomers in our assay. Thus, in a defined concentration, the 3',5'-capped, phosphorothioate-substituted oligomers seemed to remain on the cell surface, and they may be digested by nucleases in an endonucleolytic manner, rather than by exonucleolytic cleavage. In addition, the complete phosphorothioate oligomers (S-ODNs-*rev* or *tat*) showed anti-HIV activity, and which was probably due mainly to the relative resistance of the S-ODNs to nucleases, which kept them intact relative to the SO-ODNs and allowed them to reach and to remain at the target site. The anti-HIV activity of the antisense oligomers is influenced by the resistance of the oligonucleotides to nucleases, rather than to the stability of the RNA-DNA duplexes.[22]

The *tat* protein requires a specific sequence, in the 5'-end LTR promoter, termed the *TAR* element, for HIV-1 replication.[23,24] The target sequences of the antisense S-ODNs-*TAR*-1, -*TAR*-2, and -*TAR*-3 are 1–20, 15–34, and 40–59 in the 5'-terminal LTR region, respectively, but

Table 1. Comparison of anti-HIV activities of the phosphorothioate oligodeoxyribonucleotides

Oligomer[a]	Inhibitory effect (%)[b]					Cytotoxicity (%)[c]				
	0.8	4	20	100	500	0.8	4	20	100	500
	X $10^{-3}\mu$M					X $10^{-3}\mu$M				
SO-ODNs-*rev*-sa	0	0	0	0	2	0	0	0	0	5
S-ODNs-*rev*-ts	10	58	100	100	93	0	0	0	0	0
S-ODNs-*rev*-sa	8	24	100	100	100	6	0	0	0	0
S-ODNs-*tat*-sa	10	15	95	100	99	1	0	0	0	0
S-ODNs-*pol*-sa	7	66	64	92	66	40	8	34	6	26
S-ODNs-*gag*-ls	15	60	93	92	100	0	0	4	4	21
S-ODNs-*rev*-ran	38	59	70	71	0	0	0	3	8	95
S-ODNs-*rev*-sa-sen	31	50	70	69	65	0	0	0	0	24
S-ODNs-*rev*-sa-8mis	25	60	65	71	70	0	0	0	0	0
S-ODNs-*rev*-sa-16mis	38	59	62	67	73	0	0	0	0	20
S-ODNS-*TAR*-1	6	14	88	93	67	6	0	0	0	30
S-ODNS-*TAR*-2	19	30	59	78	76	6	20	31	14	29
S-ODNS-*TAR*-3	6	88	74	77	82	0	0	0	11	16

[a] The antisense sequences are described in Fig. 1.
[b] The inhibitory effect percentage of HIV-1 infected cells represents the complete protective effect by the oligomer, without cytotoxicity to MT-4 cells.
[c] Cytotoxicity of oligomers represents the percentage reduction of viable cell numbers in mock-infected MT-4 cells. Both inhibition and cytotoxicity assays were performed with the MTT assay described in the Experimental Section. Values are means of duplicate determinations.

each antisense S-ODNs-*TAR* elicited weak anti-HIV activity (88% inhibition by *TAR*-1, 59% by *TAR*-2, and 74% by *TAR*-3 at 0.02 μM, but toxic) (Table 1) This suggests a marked decrease in the ability of the antisense oligonucleotides, S-ODNs-*TAR*-1, -*TAR*-2, and -*TAR*-3, to bind to the *TAR* RNA, because of the spanning sequences on the three bulges (GGUCUCU, 1–7; UUAGACCAG, 12–20; AGAUCUGAG, 19–27) and the very short loop sites (CUGGGA, 29–35). The former binds to the *tat* protein and the latter to a 68 kD cellular protein.[25] Perhaps the secondary or tertiary RNA structures would not readily be susceptible to the antisense hybridization at the initiation of transcription or during mRNA processing.

Included in this series were three different phosphorothioate derivatives containing the *rev* sense sequence (S-ODNs/sen), a random sequence (S-ODNs/ran), and mismatched sequences (S-ODNs/8 mis and S-ODNs/16 mis). These compounds showed less activity than the antisense phosphorothioates at 0.02–0.1 μM, and at 0.5 μM were relatively toxic (Table 1). This finding differs from those of Matsukura *et al.*[9] However, only the antisense sequence of the phosphorothioate oligomers inhibited viral expression without toxicity, supporting the notion of the sequence-specific regulation of viral expression of HIV in chronically infected cells.

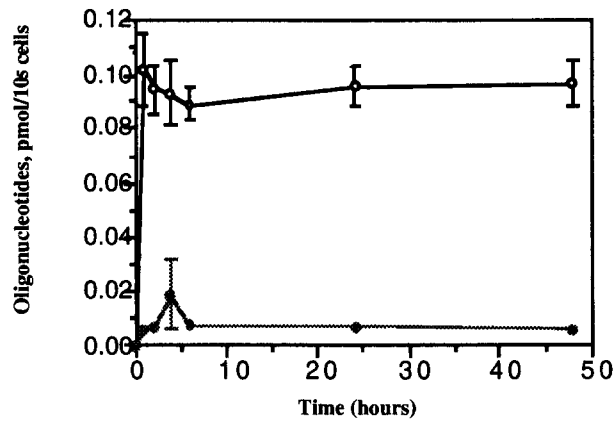


Figure 2. Cellular uptake of the S-ODNs-*rev*-ts (5'-TsCsTsCsCsGsCsTsTsCsTsTsCsCsTsGsCsCsAsT-3'○,) and the SO-ODNs-*rev*-ts (TsCsTCCGCTTCTTCCTGCCsAsT-3',●) in Molt-4 cells. Radioactive counts associated with cell lysates are expressed as pmol 10^{-5} cells over time. Vertical bars represent standard deviations.

The lengths of the phosphorothioate oligonucleotides were studied and a target site on HIV-1 RNA with a

splice acceptor site of the HIV-1 *rev* gene was used (Fig. 3). The phosphorothioate oligonucleotide with a chain length of 15 (S-ODNs-*rev*-sa-15) was as active as the 20-mer phosphorothioate oligomer (S-ODNs-*rev*-sa-20), but the phosphorothioate 10-mer (S-ODNs-*rev*-sa-10) had no activity. Of particular interest was S-ODNs-*rev*-sa-15, which had activity similar to that of the phosphorothioate 20-mer (S-ODNs-*rev*-sa-20). However, oligomer chain length had a greater effect on antiviral activity, since longer compounds were more potent. Specifically targeted antisense-phosphorothioate oligonucleotides, such as *rev* or *tat*, may be useful for treating HIV-infected patients.

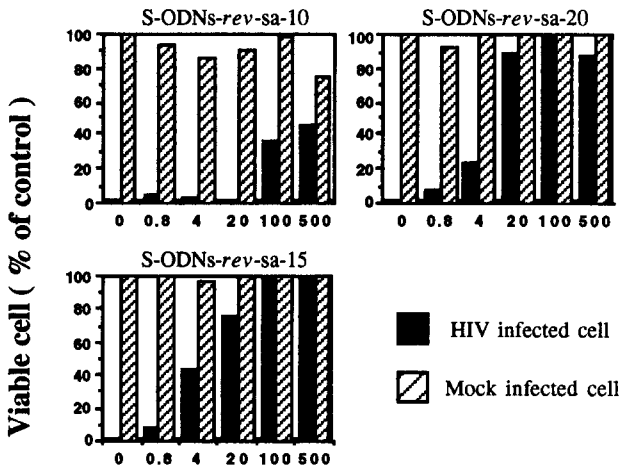


Figure 3. Effect of the chain lengths of oligonucleotides on the ability of an antisense phosphorothioate oligonucleotide (S-ODNs-*rev*-sa) to inhibit HIV-1 replication.

Non-sequence-specific inhibition of viral expression in HIV-1 infected cells

The adenosine, thymidine, and guanosine 20 mers and cytidine 15, 20, and 28 mers were tested for their comparative anti-HIV activity in acutely HIV-1 infected cells (Fig. 4). In this series, the homooligonucleotide phosphorothioates (S-dC_{28} and S-dC_{20}) had activity of the same order as the antisense phosphorothioate 20-mer (S-ODNs-*rev*-sa-20) at a concentration of 0.1 μM. However, at 0.5 μM, it showed low activity and some toxicity. On the other hand, S-dC_{15} was less active than the 20-mers (S-dC_{28} and S-dC_{20}). This observation is somewhat different from a previous description of homooligoonucleotide phosphorothioates.[9] Furthermore, the anti-HIV activity of the homooligomers S-dA_{20} and S-dT_{20} was also tested in acutely HIV-1 infected cells. S-dA_{20} and S-dT_{20} were both somewhat more active at all concentrations, and they inhibited up to 75% at concentrations from 0.1 to 0.5 μM without toxicity. These results did not indicate any correlation of the inhibitory activity with the G + C content.

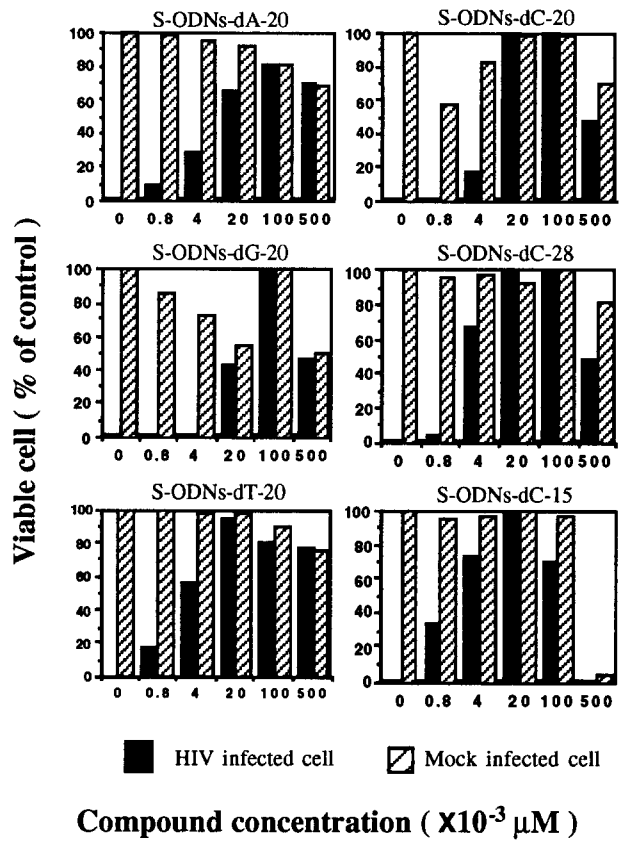


Figure 4. Effects of the chain lengths and non-sequence-specific homo-oligonucleotide phosphorothioates on HIV-1 inhibition.

Thus, homooligomers appear to behave differently from heterooligomers, and may inhibit HIV by some mechanism other than by antisense competitive hybridization. Homopolymers are better substrates than heteropolymers for reverse transcriptase.[26] We also looked for similar replication inhibition by binding to the reverse transcriptase rather than to the mRNA.[27,28] Consequently, the phosphorothioate homooligomers might be competitive inhibitors of reverse transcriptase or DNA polymerase. While the antiviral properties of the homooligomers are worth further consideration, they were less selective in their anti-HIV activity and were potentially more toxic than the antisense oligonucleotides.

Antisense oligonucleotides enter MOLT-4 cells, hybridize with complementary segments of RNA or DNA, and selectively inhibit viral replication and expression. In cells infected with HIV, perfectly matched phosphorothioate oligomers were more effective inhibitors than sense-, random-, and mismatched oligomers or homooligomers. A chain length of 15–20 mer units was optimal under our tissue culture conditions. They thus reinforce the concept that specific base pairing is a crucial feature of oligonucleotide inhibition of the human immunodeficiency virus.

Experimental

Abbreviations: HIV, human immunodeficiency virus; S-ODNs, phosphorothioate oligodeoxyribonucleotides; SO-ODNs, 3',5'-capped phosphorothioate oligodeoxyribonucleotides; HTLV-III, human T-cell lymphotropic virus type III.

Synthesis and purification of the phosphorothioate oligonucleotide analogues

The phosphorothioate oligonucleotide analogues (S-ODNs or SO-ODNs) were prepared on a synthesizer using our new phosphate approach, and were purified by HPLC according to published procedures.[29]

Sequences of the target region in the HIV genome

We used five genes [*gag*,[30,31] *pol*,[32] *rev*,[33,34] *tat*,[33,35] and *tar*[36]] as targets for antisense interruption of viral gene expression. To clarify the sequence specificity, we tested the phosphorothioate oligomers containing the *rev* sense sequence (S-ODNs/sen), *rev* mismatched sequences (S-ODNs-*rev*-sa-mis), six different gene antisense sequences [S-ODNs-*gag*-leader sequence (ls), *pol*-splice acceptor (sa), *rev*-sa, *rev*-translation start site (ts), *tat*-sa, *tar*], a random sequence with the same base composition as *rev*-sa (S-ODNs/ran), and 15–28 mer homo-oligomers [S-dC_{28}, S-dC_{20}, S-dA_{20}, S-dT_{20}, S-dG_{20}, and S-dC_{15}; Fig. 1]

Cell lines

The human T lymphotropic virus type I (HTLV-I)-positive human T cell line, MT-4, was subcultured twice weekly at a density of 3×10^5 cells μL^{-1} in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU μL^{-1} penicillin, and 100 mg mL^{-1} of streptomycin.

Virus

The HTLV-IIIB strain was used in the anti-HIV assay. The virus was prepared from the culture supernatants of MOLT-4/HTLV-IIIB cells, which were persistently infected with HTLV-IIIB. HIV stocks were titrated in MT-4 cells as determined by 50% tissue culture infectious doses ($TCID_{50}$) and plaque forming units, and stored at –80 °C until use.

Anti-HIV assay

The anti-HIV activity of test compounds in a fresh, cell-free HIV infection was determined as protection against HIV-induced cytopathic effects (CPE). Briefly, MT-4 cells were infected with HTLV-IIIB at a multiplicity of infection (MOI) of 0.01. HIV-infected or mock-infected MT-4 cells (1.5×10^5 µL, 200 µL) were placed into 96 well microtiter plates and incubated in the presence of various concentrations of test compounds. The dilution ranged from one to five-fold and nine concentrations of each compound were examined. All experiments were performed in triplicate. After a 5-day incubation at 37 °C in a CO_2 incubator, the cell viability was quantified by a calorimetric assay that monitored the ability of the viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to a blue formazan product. The absorbances were read in a microcomputer-controlled photometer (Titertek Multiskan®; Labsystem Oy, Helsinki, Finland) at two wavelengths (540 and 690 nm). The absorbance measured at 690 nm was automatically subtracted from that at 540 nm, to eliminate the effects of non-specific absorption. All data represent the mean values of triplicate wells. These values were then translated into percentage cytotoxicity and percentage antiviral protection, from which the 50% cytotoxic concentration (CC_{50}), the 50% effective concentration (EC_{50}), and the selectivity indexes (SI) were calculated.[37,38]

3'-End labelling and cellular uptake

The 3'-ends of the S-ODNs-*rev*-ts and the SO-ODNs-*rev*-ts were labelled with [α-^{32}P]dATP and terminal deoxynucleotidyl transferase, then purified using a Quick spin column (G-25). MOLT-4 cells were diluted to 1×10^5 cells ml^{-1} in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and dispensed into 24 multi-well plates. After 48 h at 37 °C in a CO_2 incubator, the RPMI-1640 medium was replaced. The cells were further incubated with the 3'-end labelled oligomers for the stated periods. After washing the cells four times with chilled phosphate buffered saline (PBS), the cells were pelleted by centrifugation and lysed in 0.5 ml of 1% sodium dodecyl sulfate (SDS). The cellular uptake of [α-^{32}P]dATP was measured in a β-scintillation counter.

Acknowledgements

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