Pergamon

Biochemical Pharmacology, Vol. 47, No. 10, pp. 1821–1826, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0029-K

INHIBITION OF THE *IN VITRO* INTEGRATION OF MOLONEY MURINE LEUKEMIA VIRUS DNA BY THE DNA MINOR GROOVE BINDER NETROPSIN

SANDRINE CARTEAU, JEAN FRANCOIS MOUSCADET, HÉLÈNE GOULAOUIC, FRÉDÉRIC SUBRA and CHRISTIAN AUCLAIR*

Laboratoire de Physicochimie et Pharmacologie des Macromolécules Biologiques, CNRS URA 147, Institut Gustave Roussy, rue Camille Desmoulins, 94801 Villejuif, France

(*Received* 15 *October* 1993; *accepted* 10 *January* 1994)

Abstract—In search of potential inhibitors of integration of retroviral DNA into host cells genome, we have investigated the effect of the external DNA binder netropsin on the *in vitro* insertion of long terminal repeat (LTR) ends of Moloney murine leukemia virus (M.MuLV) as catalysed by integrase purified from baculovirus strain expression vector. In agreement with the preferential binding of netropsin to A + T rich sequences, footprinting experiments have shown that this drug selectively binds to the 5′-TTTCAT LTR end sequence which is included in the DNA binding site of integrase. This feature results in the potent inhibition of both reactions involved in the insertion process, namely, nucleolytic cleavage and strand transfer. The relation between netropsin binding to A + T rich region of M.MuLV LTR end and inhibition of insertion is strongly suggested from the inability of the drug to inhibit the insertion of HIV U3 LTR end which displays a G + C rich sequence. Selective inhibition of integration of viral DNA appears to be feasible using drugs recognizing LTR end sequences.

Key words: leukemia; virus; HIV; integration; integrase; netropsin

One of the key steps in the cycle of retroviruses such as M.MuLV† and HIV is the insertion of a DNA copy of the RNA genome into the host cell DNA (for a general review, see Ref. 1). The integration is necessary for the expression of viral proteins and is thus considered as the principal characteristic of the retroviruses. The integration event is catalysed by a single viral protein, the IN [2, 3], which is one of the products of the *pol* gene [4, 5]. The first IN-mediated event leading to the integration of the viral HIV-1 DNA consists in the removal of the two nucleotides TG from the 3′ ends of the LTRs. This processing is followed by the cleavage of the target DNA and ligation of the processed LTR end to the 5′ ends of the cut DNA [6–8]. *In vitro* experiments using M.MuLV model integration substrates [9–13] have shown that the terminal 13 base pairs located at the ends of the LTRs are sufficient for binding of the IN protein and are required for efficient integration and that 5′-TCTTTCA-3′ is the sequence within the 13 base pairs that is primarily responsible for correct integration. This sequence can therefore be considered as a potential target for selective inhibition of integration by suitable DNA binding ligands. Among molecules displaying such properties, the bis-pyrrolecarboxamide derivative netropsin [14] (Fig. 1), which recognizes A + T-rich sequences, appears to be a possible candidate. Along this line, we previously reported that a hybrid molecule composed of a bis-pyrrolecarboxamide chain linked to the intercalating chromophore oxazolopyridocarbazole [15] inhibited the replicative cycle of M.MuLV at a post retro-transcriptional step [16]. We demonstrate in the present report that netropsin selectively binds to M.MuLV LTR ends and inhibits the *in vitro* integration process of LTR fragments as catalysed by purified IN protein. This result supports the feasibility of targeting viral DNA LTR ends with a view to impairing viral integration.

* Corresponding author.

† Abbreviations: M.MuLV, Moloney murine leukemia virus; HIV, human immunodeficiency virus; LTR, long terminal repeat; IN, integrase.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems Model 381A DNA synthesizer. The sequences synthesized were the following: LTR ends from M.MuLV: LTRa, 5′-AATGAAAGACCCCACCTG; LTRb, 5′-CAGGCTGGGGTCTTTCATT. Modified M.MuLV LTR used in footprinting experiments (U3 end): LTRc; 5′-GTCAGTCGAATGAAAGACCCCCGCTGACGGGTAGT; LTRd, 5′-ACTACCCGTCAGCGGGGGTCTTTCATTCGACTGAC. LTR ends from HIV-1: U5a, 5′-ACTGCTAGAGATTTTCCACAC; U5b, 5′-GTGTGGAAAATCTCTAGCAGT; U3a: 5′-ACTGGAAGGGCTAATTCACTC; U3b: 5′-GAGTGAATTAGCCCTTCCAGT. The M.MuLV and HIV-1 DNA substrates, which correspond to the LTR U3/U5 ends of M.MuLV DNA and the U5 end of HIV-1 DNA, were made by labeling the 5′ end of the 21-mer oligonucleotide which matches the corresponding LTR ends with ^{32}P and annealing with its complement. One hundred nanograms of the oligonucleotide to be labeled were phosphorylated by polynucleotide kinase in the presence of 100 μCi of [γ-^{32}P]ATP (sp. act., 3000 Ci/mmol) in a reaction

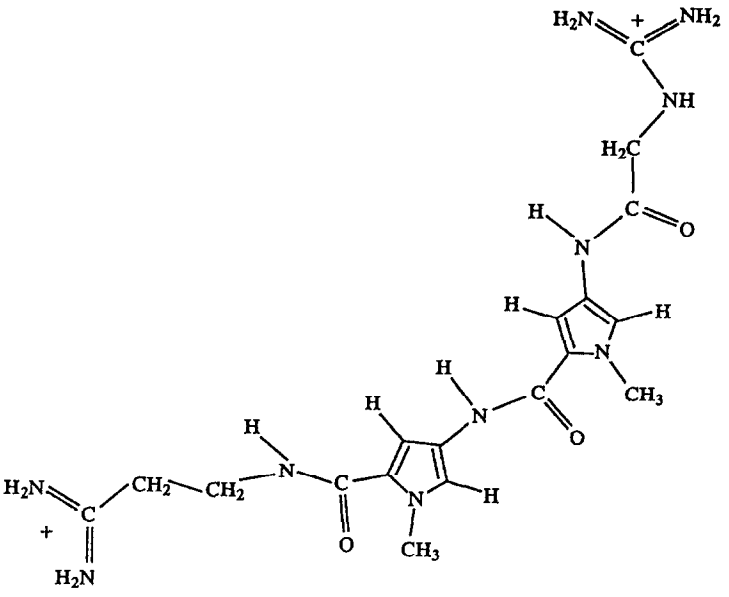

Fig. 1. Structure of netropsin.

volume of 15 μL. EDTA was then added to a final concentration of 25 mM, and the polynucleotide kinase was inactivated by heating to 65° for 15 min. NaCl was added to a final concentration of 0.1 M with 100 ng of the complementary unlabeled strand, in a total volume of 40 μL. The mixture was heated to 90° for 2 min and the DNA was annealed by slow cooling. Unincorporated nucleotide was removed by passage through Sephadex G-10.

Footprinting experiment

DNase I footprinting was performed in buffer containing 20 mM Tris–HCl pH 7.0, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 60 ng plasmid pSP65 and 10 nM oligonucleotide. Netropsin was added to the reaction mixture and incubated at 4° for 1 hr. Digestion was started by the addition of DNase I (3 U/mL) and stopped after 2.5 min by adding EDTA (10 mM), sodium acetate (0.3 M) and carrier tRNA (5 μg). Products of reaction were subsequently precipitated with ethanol, dried and resuspended in formamide/EDTA gel loading buffer.

Gel electrophoresis. DNA fragments were separated by electrophoresis in gels composed of 18% polyacrylamide containing 8 M urea and Tris/borate/EDTA buffer. After 3 hr of electrophoresis at 1500 V, the gels were directly subjected to autoradiography at −70° with an intensifying screen.

Densitometry. Autoradiographs from the footprinting experiments were analysed using a Joyce scanning microdensitometer to produce profiles from which the relative intensity of each band was measured. These intensities were calculated in terms of fractional cleavage (f) = A_i/A_t where A_i is the area under band i and A_t the mean of the sum of the areas under all bands in the corresponding gel lane. Protection or enhancement in the presence of drug were expressed as per cent of fractional cleavage compared with the control.

Oligonucleotide sequencing. Modified Maxam-Gilbert sequencing reactions were used to generate T ladders of end-labeled DNA [17]. Labeled DNA (9 μL) was heated to 90° for 2 min, cooled quickly to room temperature, mixed with 1 μL of 3 mM $KMnO_4$, incubated for 7 min at room temperature and finally quenched with 1 μL allyl alcohol. The mixture was then treated for 15 min with 1 M pyrrolidine at 90°, dried and resuspended in formamide loading buffer.

Baculovirus strain

IN protein was expressed in *Spodoptera frugiperda* (SF9) cells infected with a recombinant baculovirus Autographa California Nuclear Polyhedrosis Virus (AcMnPV) that contained the entire coding sequence of M.MuLV IN protein under the transcriptional regulation of the viral polyhedrin promoter. The virus strain was provided by R. Craigie (National Institutes of Health).

Purification of soluble M.MuLV IN protein

IN protein was purified as described by Craigie *et al.* [2].

Purification of soluble HIV IN protein

IN protein from HIV-1 expressed in *Escherichia coli* was purified according to Sherman and Fyfe [18] with minor modifications as described previously [19].

M.MuLV and HIV-1 DNA integration reactions

The M.MuLV DNA integration reaction mixture (20 μL) contained 20 mM HEPES pH 7.2, 10 mM $MnCl_2$, 20% (w/v) glycerol, 10 mM DTT, 0.1 mg/mL BSA, 5 ng M.MuLV DNA and approximately

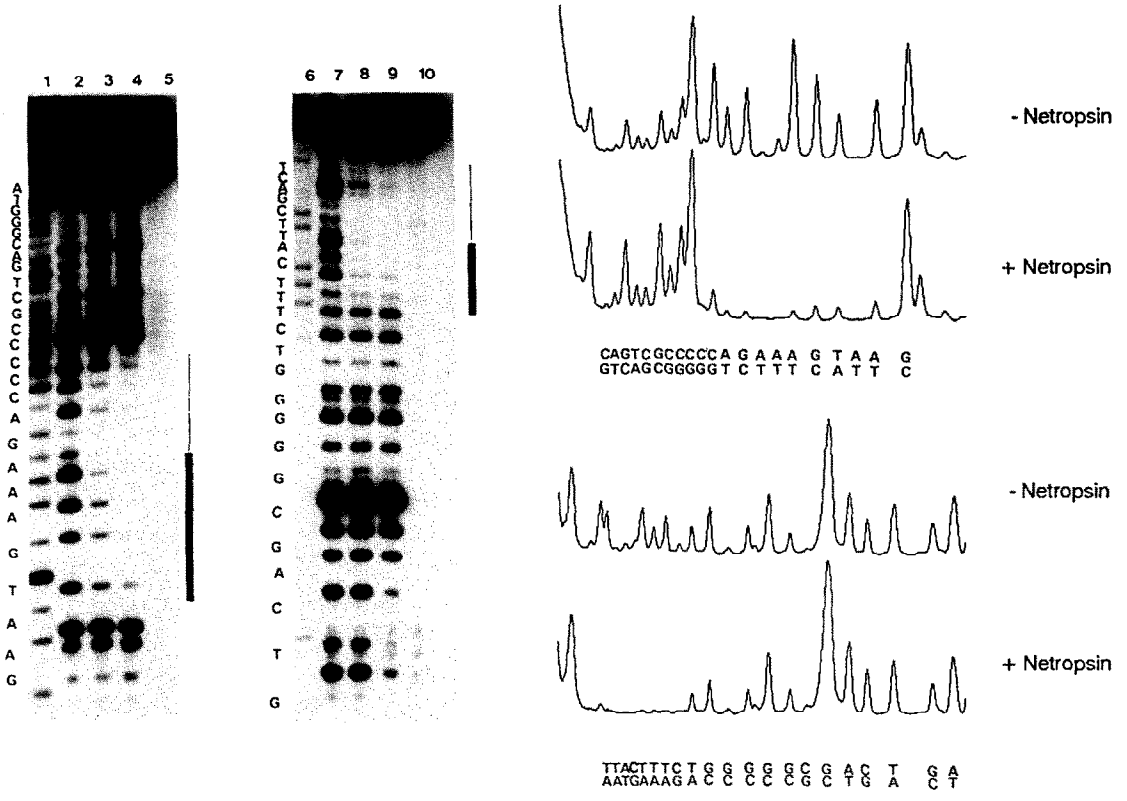

Fig. 2. Footprinting assays using DNase I in the presence of netropsin. The used oligonucleotide mimics the extremity of the M.MuLV LTR U3 sequence which is labeled on the 5′ end of the LTRc (lanes 1 to 5) or LTRd (lanes 6 to 10) strand. Lanes 1 and 6, chemical cleavage of the thymines. Lanes 5 and 10, oligonucleotides without DNase I. Lanes 2 to 4 and 7 to 9, DNase I cleavage without (2 and 7) or with netropsin 10 μM (3 and 8) or 50 μM (4 and 9). The lines indicate the protected sites and the thick lines indicate the common protected site on both strands. Densitometric scans of autoradiograms without or with 50 μM netropsin are indicated on the right of the figure.

0.25 pmol of M.MuLV IN protein. The HIV-1 DNA integration reaction mixture (20 μL) contained 20 mM HEPES pH 7.0, 10 mM $MnCl_2$, 10% (w/v) glycerol, 10 mM DTT, 0.1 mg/mL BSA, 5 ng of U3 or U5 HIV-1 DNA and approximately 0.5 pmol of HIV-1 IN protein. The reactions were carried out at 30° for 150 min. The reactions were then stopped by addition formamide, EDTA, xylene cyanol and bromophenol blue to final concentrations of 30% (w/v), 10 mM, 0.08% and 0.08%, respectively. Autointegration products were separated on an 18% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Footprinting experiments

Footprinting experiments [20, 21] as well as NMR spectroscopy [22] on netropsin have shown that binding sites are A + T rich and 5–7 bases long. The crystal structure of netropsin bound to a dodecamer containing AATT has elucidated the binding mode [23] and further work has confirmed that netropsin recognizes A + T-rich regions which are at least four base pairs long [24]. Presently, there is general agreement that sequences of 4 bases long composed of non alternating bases such as 5→3′TTTA and AATT are good binding sequences for the drug [25]. The LTR end of M.MuLV DNA displays two A + T rich sequences of 3 base pairs separated by a single GC base pair: 5′-CTTTCATT. We first investigated the binding of netropsin to these sequences using DNase I footprinting experiments performed on both strands of the duplex. In order to minimize edge effects we synthesized duplex DNA matching the 21 terminal bases of the LTR ends to which a short random sequence of 5 bases (CGACT) was added, yielding the following end sequence: 5′-...CTTTCATTCGACT. Typical DNase I digestion patterns for the M.MuLV U3 LTR end DNA duplex in the presence of increasing concentrations of netropsin are presented in Fig. 2. The protected site is clearly visible and centered around the A + T-rich region. The densitometric scans performed on both strands show that the protection against enzyme cleavage is located at the 5′-TTTCAT sequence despite the presence of the extra GC base pair between the two A + T-rich clusters. This can be explained by the fact that both 5′-TTT and 5′-AT

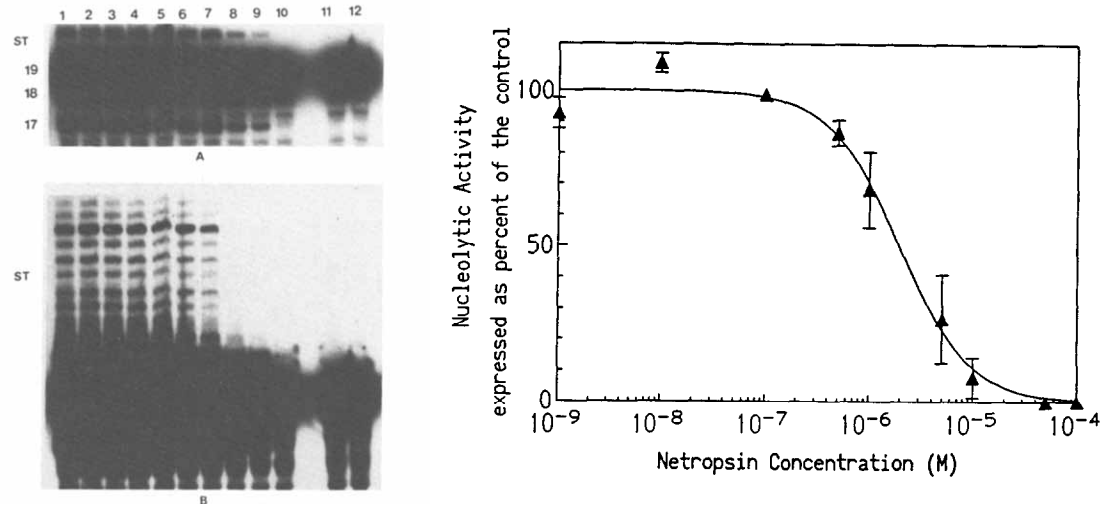

Fig. 3. The effect of netropsin on the *in vitro* autointegration of the M.MuLV DNA. M.MuLV oligonucleotide was incubated for 150 min at 30°C with M.MuLV IN in the presence of increasing concentrations of netropsin. 11, control without IN; 12, control with 10^{-4} M of netropsin without IN. 1–10, oligonucleotide with IN (1) in addition to 10^{-9} (2), 10^{-8} (3), 10^{-7} (4), 5×10^{-7} (5), 10^{-6} (6), 5×10^{-6} (7), 10^{-5} (8), 5×10^{-5} (9) or 10^{-4} M (10) of netropsin. Panel A (indicative for the endonucleolytic cleavage product) shows a shorter exposure of the gel presented in panel B which displays the strand transfer products (ST). The curve describes the percentage of the endonucleolytic cleavage activity of the integrase (densitometric analyses of the spot named 17-mer in the panel A) with respect to the control.

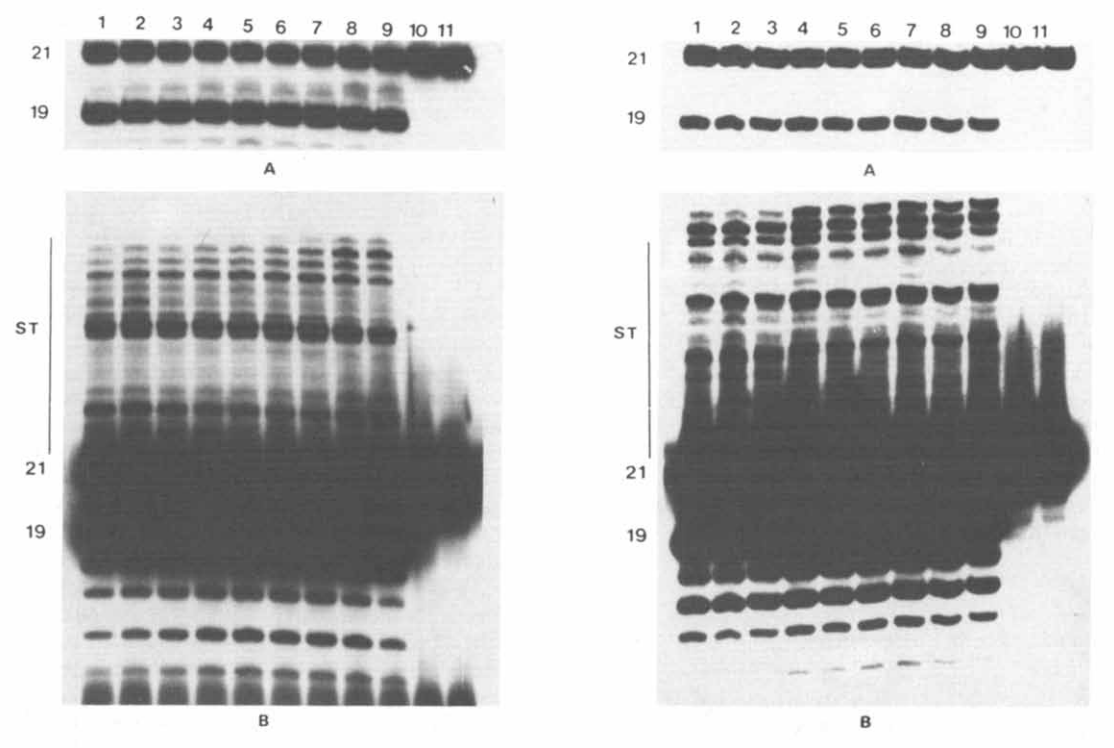

Fig. 4. Effect of netropsin on the *in vitro* autointegration of U5 (left figure) or U3 (right figure) LTR extremities of HIV. Oligonucleotides were incubated for 150 min at 30°C with HIV-1 IN in the presence of increasing concentrations of netropsin. 11, control without IN; 12, control with 10^{-4}M of netropsin without IN. 1–10, oligonucleotide with IN (1) in addition to 10^{-8} (2), 10^{-7} (3), 5×10^{-7} (4), 10^{-6} (5), 5×10^{-6} (6), 10^{-5} (7), 5×10^{-5} (8) or 10^{-4}M (9) of netropsin.

are highly favorable for netropsin binding [25]. Such protection at sequences like 5′-TTTCTT has been described already using EDTA-Fe(II) as DNA cleaving reagent [15].

Effect of netropsin on LTR insertion

Purified M.MuLV IN protein catalyses the overall integration reaction, namely the processing and strand transfer steps using the LTR ends oligonucleotide as substrate. In agreement with previous observations, oligonucleotides matching M.MuLV LTR termini can be cleaved in the presence of the IN protein and subsequently inserted into another LTR molecule (Figs 3 and 4, panels A and B). This process leads to the appearance of various new labeled products including the oligonucleotide resulting from the processing event as well as products longer and shorter than the initial oligonucleotide resulting from the strand transfer event. The longer products generated following the strand transfer reaction are derived from the ligation of the 3′ end of the processed LTR substrate to the 5′ end of a nick made in another LTR molecule acting as target. The shorter products are derived from the corresponding unjoined fragments of the target DNA strand. Figure 3 shows that the presence of increasing concentrations of netropsin in the assay medium results in a marked inhibition of the overall integration process. The inhibitory action occurs on both the processing and the strand transfer steps with an identical IC_{50} value. This inhibition probably results from the inability of the IN protein to bind to its substrate DNA–netropsin complex. This assumption is supported by the fact that netropsin and the related compound distamycin A have been found to selectively interfere with various proteins recognizing A + T-rich DNA sequences, including the restriction enzyme EcoRI [15], the transacting nuclear factors of the human HLA-DRa gene which bind to a GTATA site [26], and an *Antennapedia* homeodomain peptide recognizing a 5′-ATTA sequence [27]. However, potent inhibition of *in vitro* viral DNA integration can be achieved at micromolar concentrations by unselective DNA binding ligands such as intercalating agents [28]. In order to exclude a possible unselective interaction with the duplex LTR DNA independently of the presence of the A + T containing sequence or the IN protein, we investigated the effect of netropsin on the integration of the U5 and U3 LTR of HIV-1 DNA as catalysed by the purified HIV-1 IN protein expressed in *E. coli*. Both U5 and U3 LTR ends display a sequence different from the M.MuLV LTR which is characterized by the absence of an A + T-rich sequence in the region required for integration (see Materials and Methods). Figure 4 shows that in this experimental model the presence of netropsin up to 100 μM did not result in any inhibitory action on integration of either the U3 or U5 HIV LTR ends. This strongly suggests a direct relationship between sequence recognition and the inhibitory effect of the drug.

CONCLUSION

The data obtained in the present investigation indicate that the use of compounds that selectively bind to LTR end sequences involved in the integration process of viral DNA results in the inhibition of the catalytic activity of the IN protein. LTR end DNA sequences therefore appear to be suitable targets for drugs designed to inhibit the integration of viral DNA selectively. Accordingly, DNA binding ligands recognizing A + T-rich sequences may be considered as potential inhibitors for the integration of viruses such as M.MuLV, SNV, RSV and FeLV whose LTR ends display A + T clusters, as well as mobile DNA such as the IRU5 of IAP sequences. The pharmacological relevance of such an approach extended to retroviruses responsible for human diseases, such as HTLVI and HIV, requires the design of molecules able to read various types of duplex sequences. Along this line, the targeting by triplex forming oligonucleotides (TFO) of the HIV-1 IN binding site located at the end of U3 LTR and composed of a short homopurine/homopyrimidine tract could be a typical example of such a strategy.

Acknowledgements—This work was supported by the ANRS antiviral research program and ARC Grant No. 2035.

REFERENCES

1. Goff SR, Genetics of retroviral integration. *Annu Rev Genet* **26**: 527–544, 1992.
2. Craigie R, Fukiwara T and Bushman FD, The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. *Cell* **62**: 829–837, 1990.
3. Bushman FD, Fujiwara T and Craigie R, Retroviral DNA integration directed by HIV integration protein *in vitro*. *Science* **249**: 1555–1558, 1990.
4. Donehower LA and Varmus HE, A mutant murine leukemia virus with a single missense mutation codon in *pol* is defective in a function affecting integration. *Proc Acad Natl Sci USA* **81**: 6461–6465, 1984.
5. Panganiban A and Temin H, The retrovirus *pol* gene encodes a product required for DNA integration: identification of retrovirus int locus. *Proc Natl Acad Sci USA* **81**: 7885–7889, 1984.
6. Brown PO, Bowerman B, Varmus HE and Bishop JM, Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. *Proc Natl Acad Sci USA* **86**: 2525–2529, 1989.
7. Katzman M, Katz RA, Skalka AM and Leis J, The avian retroviral integration protein cleaves the terminal sequences of linear viral DNA at the *in vivo* sites of integration. *J Virol* **63**: 5319–5327, 1989.
8. Engelman A, Mizuuchi K and Craigie R, HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell* **67**: 1211–1221, 1991.
9. Colicelli J and Goff SP, Mutants and pseudorevertants of Moloney murine leukemia virus with alterations at the integration site. *Cell* **42**: 573–580, 1985.
10. Brown PO, Bowerman B, Varmus HE and Bishop JM, Correct integration of retroviral DNA *in vitro*. *Cell* **49**: 347–356, 1987.
11. Basu S and Varmus HE, Moloney murine leukemia virus integration protein produced in yeast binds specifically to viral att sites. *J Virol* **64**: 5617–5625, 1990.
12. Bushman F and Craigie R, Sequence requirement for integration of Moloney murine leukemia virus DNA *in vitro*. *J Virol* **64**: 5645–5648, 1990.

13. Krogstad PA and Champoux JJ, Sequence-specific binding of DNA by the Moloney murine leukemia virus integrase protein. *J Virol* **64**: 2796–2801, 1990.
14. Hahn FE, Distamycin A and netropsin. In: *Antibiotics III* (Eds. Corcoran JW and Hahn FE), pp. 79–100. Springer, New York, 1985.
15. Subra F, Carteau S, Pager J, Paoletti C, Auclair C, Mrani D, Gosselin G and Imbach JL, Bis-(Pyrrolecarboxamide) linked to intercalating chromophore oxazolopyridocarbazole (OPC): selective binding to DNA and polynucleotides. *Biochemistry* **30**: 1642–1650, 1991.
16. Subra F, Mouscadet JF, Lavignon M, Roy C and Auclair C, Inhibition of the Moloney Murine Leukemia Virus cycle at a post reverse transcription step by the netropsin-intercalating molecule netropsin–oxazolopyrido–carbazole. *Biochem Pharmacol* **45**: 93–99, 1993.
17. Williamson JR and Celander DW, Rapid procedure for chemical sequencing of small oligonucleotides without ethanol precipitation. *Nucleic Acids Res* **18**: 379, 1990.
18. Sherman PA and Fyfe JA, Human immunodeficiency virus integration protein expressed in *Escherichia coli* possesses selective DNA cleaving activity. *Proc Natl Acad Sci USA* **87**: 5119–5123, 1990.
19. Carteau S, Mouscadet JF, Goulaouic H, Subra F and Auclair C, Quantitative *in vitro* assay for HIV DNA integration. *Arch Biochem Biophys* **300**: 756–760, 1993.
20. Lane MJ, Dabrowiak JC and Vournakis JN, Sequence specificity of actinomycin D and netropsin binding to pBR322 DNA analyzed by protection from DNase I. *Proc Natl Acad Sci USA* **80**: 3260–3264, 1983.
21. Harshman KD and Dervan PB, Molecular recognition of B-DNA by Hoechst 33258. *Nucleic Acids Res* **13**: 4825–4835, 1985.
22. Sarma MH, Gupta G and Sarma RH, Netropsin specifically recognizes one of the two conformationally equivalent strands of poly (dA)-poly (dT). One dimensional NMR study at 500 MHz involving NOE transfer between netropsin and DNA protons. *J Biomol Struct Dynam* **2**: 1085–1095, 1985.
23. Kopka ML, Yoon C, Goodsell D, Pjurra P and Dickerson RE, The molecular origin of the DNA-drug specificity in netropsin and distamycin. *Proc Natl Acad Sci USA* **82**: 1376–1380, 1985.
24. Portugal J and Waring MJ, Comparison of binding sites in DNA for berenil, netropsin and distamycin. *Eur J Biochem* **167**: 281–289, 1987.
25. Ward B, Rehfuss R, Goodisman J and Dabrowiak JC, Determination of Netropsin-DNA binding constants from footprinting data. *Biochemistry* **27**: 1198–1205, 1988.
26. Gambari R, Barbieri R, Nastruzzi C, Chiorboli V, Feriotto G, Natali PG, Giacomini P and Arcamone F, Distamycin inhibits the binding of a nuclear factor to the −278/−256 upstream sequence of the human HLA-DRa gene. *Biochem Pharmacol* **41**: 497–502, 1991.
27. Dorn A, Affolter M, Müller M, Gehring WJ and Leupin W, Distamycin-induced inhibition of homeodomain-DNA complexes. *EMBO J* **11**: 279–286, 1992.
28. Carteau S, Mouscadet JF, Goulaouic H, Subra F and Auclair C, Effect of topoisomerase inhibitors on the *in vitro* HIV DNA integration reaction. *Biochem Biophys Res Commun* **192**: 1409–1414, 1993.