FUNCTION OF THE N CISTRON OF BACTERIOPHAGE LAMBDA

By Jay J. Protass and David Korn

LABORATORY OF BIOCHEMICAL PHARMACOLOGY, NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH

Communicated by Scott E. Forbush, March 18, 1966

In 1961, Campbell,[1] on the basis of his work with suppressible mutants of λ, identified 18 complementation groups which appeared to span the entire λ chromosome. These groups are arranged in alphabetical order from left to right on the vegetative λ map. Thirteen of them, A–M, lie on the left half of the λ DNA molecule and appear to be concerned with "late" maturation and morphogenetic functions. The remaining five cistrons, N–R, lie on the right half of the λ genome and are concerned with "early" functions necessary for the initiation of vegetative phage development.[1, 2]

Of the various λ cistrons that have been identified genetically, specific functions have thus far been assigned only to three: (1) The C_I region, in the right half of λ DNA,[3] determines the synthesis of the λ repressor and, possibly, of the site(s) on which this repressor acts.[4, 5] (2) Cistron R has been shown unequivocally to be the structural gene for endolysin.[1, 6, 7] (3) In 1964, Radding proposed[8, 9] that cistron N might be the structural gene for the λ-directed DNA-exonuclease.[10] This proposal was based upon the observation that among a number of defective λ lysogens studied, only those containing prophages with a mutation in N failed to produce exonuclease.

Radding's experiments did not conclusively establish that the failure of N mutants to synthesize exonuclease was a direct, rather than an indirect, consequence of the N lesion. One could choose between these alternatives by determining whether absence of exonuclease is the sole consequence of N mutations or, in contrast, whether N mutants also fail to perform additional functions which are known to be directly controlled by other phage loci. We have attempted to explore this problem by examining the ability of a number of λ mutants to synthesize the only two enzymes known to be associated with λ development, exonuclease and endolysin.

The data to be presented in this paper demonstrate the following: (1) In confirmation of the observations of Radding and Campbell, only N mutants fail to synthesize exonuclease; R mutants do not make endolysin. (2) Early mutants mapping in cistrons O, P, and Q all produce low but significant amounts of endolysin. However, none of the five different N mutants tested synthesized detectable levels of endolysin.

In itself, the fact that N mutants, uniquely among all those tested, fail to produce *either* of the two known λ-directed enzymes questions the validity of the proposal that N is the structural gene for exonuclease. This finding, together with the observations of others to be discussed subsequently, attests to the extreme defectiveness of N mutants and leads us to suggest the hypothesis that N may be a regulatory locus that controls the transcription of the early cistrons of λ.

Materials and Methods.—Bacterial and phage strains: The permissive strain of *E. coli*, C600, and the wild-type lysogen, C600 (λ^+), were from stocks maintained in this laboratory. The nonpermissive strain, W3350, was obtained from Dr. M. B. Yarmolinsky. C600 lysogens containing

various suppressible (sus) mutants of λ were originally prepared by Dr. A. Campbell and were given to us by Dr. A. Weissbach. Stocks of λ sus phage were prepared by induction of the appropriate permissive lysogen, and these phages were then used in high-multiplicity infection of W3350 to prepare stocks of defective W3350 lysogens. The strains W3350 (λ sus N_{53}) and W3350 (λ sus N_{96A}) were gifts of Mr. J. Zissler and Dr. A. Campbell. T_{11} (λT_{11}), a K12 lysogen containing the λ mutant that has been shown to produce excessive amounts of exonuclease,[9] was derived from the collection of Dr. C. R. Fuerst and given to us by Dr. M. B. Yarmolinsky. Stocks of λC_{72} (C_I), λC_{68} (C_{II}), and λC_{67} (C_{III}) were obtained from Dr. V. Bode. The lysogen C600 (λC_{III}) was prepared in this laboratory. Phage λC_{17} (isolated by Dr. F. Jacob) was obtained from Dr. M. B. Yarmolinsky, and λvir from Dr. A. D. Kaiser.

Techniques of cell growth, mitomycin induction, and phage assay, and composition of media have all been previously described.[11] At indicated times after the onset of mitomycin treatment of bacterial cultures, aliquots were rapidly cooled to 0°C and the cells harvested by centrifugation.

Preparation of crude extracts: All operations were performed at 0–4°C. The cell pellet was suspended at a concentration of 6–8 × 10^9/ml in 1–2 ml of 0.01 *M* Tris-chloride buffer, pH 7.5. One gram of Superbrite no. 120 glass beads (Reflective Products Division, Minnesota Mining and Manufacturing Co.) was added, and the suspension was homogenized for three separate periods of 20 sec each with a Sorvall Omnimixer fitted with a microhomogenizer attachment. Beads and debris were removed by centrifugation at 10,000 *g* for 10 min, and the resulting supernatant comprised the crude extract. Protein concentration of extracts was measured by the procedure of Lowry *et al.*[12]

Enzyme assays: The λ-directed DNA exonuclease was assayed in crude extracts by the method of Korn and Weissbach.[10] The substrate was H^3-K12(λ)-DNA, prepared as previously described.[13] In this study the unit of nuclease activity is defined as the release in acid-soluble form of 1000 cpm/min (1.5 mμmole of nucleotide phosphorus). Specific activity is defined as units per mg of protein. Endolysin was assayed by a modification of the method of Sekiguchi and Cohen,[14] described in detail previously.[15] The reaction was carried out at room temperature in a Beckman DU spectrophotometer employing cuvettes with a light path of 10 mm. The unit of endolysin activity is defined as a decrease of 0.100 optical density unit per minute at 650 mμ. All enzyme assays were carried out under conditions of proportionality with respect to time and protein concentration.

Results.—Under our experimental conditions, mitomycin induction of the wild-type lysogen W3350 (λ$^+$) results in lysis by 70–80 min. The kinetics of synthesis of exonuclease and endolysin in induced W3350 (λ$^+$) are depicted in Figure 1. It is

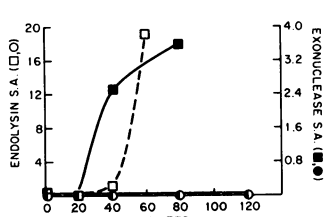

FIG. 1.—Synthesis of exonuclease and endolysin following mitomycin C treatment of W3350 (λ$^+$) and W3350 (λ sus N_7). Mitomycin C (1 μg/ml) was added to exponentially growing cells (~3 × 10^8/ml), and samples containing about 6 × 10^9 cells were withdrawn at indicated times thereafter and rapidly chilled. Extracts were prepared and tested for enzyme activities as described in *Materials and Methods*. ■, Exonuclease, W3350 (λ$^+$); ●, exonuclease, W3350 (λ sus N_7); □, endolysin, W3350 (λ$^+$); ○, endolysin, W3350 (λ sus N_7). *S.A.*, specific activity.

apparent that in accord with previous observations,[16] exonuclease begins to appear very early in the latent period and increases in amount up to the onset of lysis. In contrast, endolysin synthesis cannot be detected until 40 min have elapsed; thereafter the activity increases rapidly until lysis begins. A detailed study of the factors affecting the synthesis of these two λ-directed enzymes will be presented elsewhere.[17]

Of the various W3350 (λ sus) defective lysogens tested, all showed a pattern of exonuclease synthesis similar to that of the wild-type lysogen (Table 1, *A* and *B*, and Fig. 1), with the exception of those containing prophages with sus mutations in

TABLE 1

PRODUCTION OF ENDOLYSIN AND EXONUCLEASE BY VARIOUS λ MUTANTS FOLLOWING LYSOGENIC INDUCTION OR PHAGE INFECTION

Bacterial strain	Endolysin specific activity (Time of Assay)	Exonuclease specific activity (Time of Assay)	Lysis	Immunity loss
A	60 min	60 min		
W3350 (λ^+)	24.5	2.1	+	+
W3350 (λ sus N_7)	0.0	0.0	−	+
W3350 (λ sus N_{53})	0.0	0.0	−	+
W3350 (λ sus N_{96A})	0.0	0.0	−	+
P_{22}	0.0	0.0	−	+
T_{27}	0.0	0.1	−	+
T_{11}	0.0*	9.7*	−	
B	80 min	80 min		
W3350 (λ sus A_{11})	28.2	1.8	+	
W3350 (λ sus L_{63})	20.0	1.1	+	
W3350 (λ sus O_8)	6.0*	2.5	−	
W3350 (λ sus P_3)	3.4	1.2	−	
W3350 (λ sus Q_{21})	2.7	3.2	−	
W3350 (λ sus R_5)	0.0	2.0	−	
C	80 min	80 min		
C600 (λ^+)	15.0	2.8	+	
C600 (λ_{CIII})	22.0	2.3	+	
D	60 min	40 min		
λvir	21.0	5.5	+	
λCI	23.0	4.1	+	
λCII	19.0	5.5	+	
λC17	19.4	3.0	+	

Lysogenic induction with mitomycin C (parts *A*, *B*, and *C*) was accomplished as described in the legend to Fig. 1. In part *D*, infection of sensitive cells of *E. coli* C600 was carried out at an input multiplicity of about 5 phage/bacterium. The preparation of crude extracts and methods of assaying enzymatic activity are described in *Materials and Methods*. Loss of superinfection immunity was assayed by mixing samples of lysogens, both at time 0 and after exposure to mitomycin C for 25 min (W3350(λ^+)) or for 60 min (W3350(λdef)) with λC_I (input multiplicity, 5:1). After incubation in synthetic medium for 5 min at 37°C, the samples were centrifuged at 10,000 *g* to remove free phage, and the pellets were resuspended in fresh medium. These resuspended pellets were then assayed for loss of immunity by plating appropriate dilutions on C600 and scoring for the development of infectious centers. In the absence of superinfection none of the defective N lysogens produced infectious centers detectable at these dilutions following treatment with mitomycin C.

* Time of assay: 2 hr.

cistron N. Similarly, defective lysogens P_{22} and T_{27}, also containing lesions in N, did not synthesize detectable quantities of exonuclease. T_{11}, as Radding originally reported,[9] produced abnormally large quantities of this enzyme.

It has been generally recognized that lysogens containing early mutants of λ do not lyse following treatment with an inducing agent, and the scoring for lysis presented in Table 1, *A* and *B*, is in accord with earlier observations of others.[1, 6, 8, 9] However, when extracts were prepared from induced cultures of the various defective lysogens and were tested for endolysin activity *in vitro* (Table 1, *A* and *B*), all possessed detectable amounts of enzyme except for sus R, T_{11}, and the several N mutants (Fig. 1). Cistron R, as noted above, appears to be the structural gene for endolysin.[1, 7]

The two late mutants tested, λ sus A and λ sus L, both showed a pattern of endolysin synthesis almost identical to that of the wild-type lysogen. With three of the early mutants, λ sus O, λ sus P, and λ sus Q, we have consistently found amounts of endolysin activity ranging between 10 and 20 per cent of the wild-type level. In a study to be reported elsewhere,[17] we have obtained evidence suggesting that the

synthesis of endolysin, but not of exonuclease, is partially dependent upon λ DNA replication, and the diminished level of endolysin obtained with the mutants sus O and sus P is entirely comparable to those obtained with λ^+ under conditions that severely restrict λ DNA synthesis. The low level of endolysin produced by λ sus Q has also been observed by Joyner *et al.*[18] during their study of λ DNA and mRNA synthesis in induced defective lysogens of W3350. The finding that these early mutants of λ that do not cause lysis *in vivo* do synthesize endolysin is compatible with the finding by Groman and Suzuki[19] that endolysin in itself was not necessarily sufficient to lead to the lysis of induced K12(λ).

In contrast to these results with sus mutants O, P, and Q, it is seen (Table 1, *A* and *B*) that no detectable endolysin is present in extracts of induced lysogens containing three different sus N mutants or defective prophages P_{22}, T_{27}, or T_{11}. Under conditions of the enzyme assay, an amount of activity equal to 0.2–0.5 per cent of that in wild-type extracts would have been detectable. Mixing experiments with extracts prepared from the wild-type lysogen and from each of the early defective lysogens failed to reveal the presence of an inhibitor of endolysin activity. In another experiment, cultures of the various early defective lysogens were shaken with mitomycin C at 37°C for 2 hr. At the end of this time the cultures, which had not lysed, were treated with a few drops of chloroform[20] and incubated at room temperature for 80 min. Lysis, as indicated by a decrease in optical density of the cultures, was apparent for those lysogens containing mutants sus O, P, and Q, but not for those containing the N mutants or sus R.

To try to determine why the early mutants O, P, and Q did produce some endolysin, we investigated the possibility that these mutants might be leaky. Brooks stated[2] that λ sus mutants on the right side of the map are known to be leaky, but quantitative data on this problem are not, to our knowledge, available. The results of a relevant experiment are shown in Table 2. The stocks of λ sus phage which were used to prepare the W3350 defective lysogens were pure, showing plating efficiencies on W3350 compared to C600 of 10^{-5} to 10^{-7}. From the data in Table 2 it is apparent first that the infectious center results are misleading. For example, W3350 (λ sus R) is induced and produces a normal phage titer but cannot produce

TABLE 2

INFECTIOUS CENTER AND PHAGE PRODUCTION BY DEFECTIVE λ LYSOGENS FOLLOWING MITOMYCIN C TREATMENT

Lysogen	Infectious centers (per ml) assayed on C600	"Lysate" titer (PFU/ml) assayed on C600	"Lysate" titer as % of control	Fraction of "lysate" titer able to plate on W3350
W3350 (λ^+)	1.1×10^8	1.4×10^{10}	100	...
W3350 (λ sus N_7)	1.7×10^4	$<1 \times 10^4$	<0.0001	...
W3350 (λ sus N_{53})	2.9×10^5	$<1 \times 10^4$	<0.0001	...
W3350 (λ sus N_{96A})	$<1 \times 10^4$	$<1 \times 10^4$	<0.0001	...
T_{27}	$<1 \times 10^4$	$<1 \times 10^4$	<0.0001	...
W3350 (λ sus O_8)	2.2×10^6	7.0×10^6	0.05	$<10^{-4}$
W3350 (λ sus P_3)	9.8×10^6	1.9×10^8	1.4	$<10^{-4}$
W3350 (λ sus Q_{21})	1.7×10^6	2.6×10^8	1.9	$<10^{-4}$
W3350 (λ sus R_5)	1.9×10^6	1.3×10^{10}	93	$<10^{-6}$

Cells were grown into log phase, and at an identical optical density were treated with 1 μg/ml of mitomycin C. After 25 min an aliquot was removed from each culture, chilled, centrifuged, and the cells were resuspended for assay of infectious centers. After 120 min (the control had lysed) aliquots from all but the control culture were again removed and centrifuged. The cells were resuspended in 3 ml of 0.05 *M* Tris-chloride buffer, pH 8.0, containing 10^{-3} *M* ethylenediaminetetraacetate and 100 μg/ml lysozyme (2× crystallized muramidase, Worthington Biochemical Corp.) and incubated at room temperature for 10 min. The samples were then diluted to their original volume and centrifuged at low speed to remove debris. The supernatants comprised the "lysates" that were assayed for PFU.

infectious centers on C600, presumably because of lysozyme deficiency. Similarly, free phage are not detected in cultures of early defectives that do not lyse after 2 hr of exposure to mitomycin C unless one artificially ruptures the cells to release intracellular mature phage. If such rupture is performed, however, it is apparent that W3350 (λ sus R) does produce a normal complement of phage, and that the defectives O, P, and Q all produce very low but significant phage titers. That this leakiness is not due to reversion is shown by the fact that the phage produced by these cells do not plate on W3350 and thus retain their sus characteristics. In contrast, the defective N mutants tested by these criteria are significantly less leaky than the other early mutants. Although it is *possible* that the small amounts of endolysin produced by sus mutants O, P, and Q may be due to leakiness, this possibility in no way affects the main point of these experiments, namely, that none of the N mutants tested is able to direct the synthesis of endolysin.

Since our data indicated that N mutants, uniquely among all those studied, synthesized *neither* of the two known λ-directed enzymes, it became critical to determine whether mitomycin treatment of these mutants led to any recognizable concomitant of lysogenic induction. Accordingly, we investigated whether these defective lysogenic cells lost their immunity to the superinfecting homologous phage, λC_I, following exposure to mitomycin C. Loss of immunity could be demonstrated in all instances (Table 1, *A*, last column).

Because the N cistron has been shown to lie within the C region, between C_{III} and C_I (the map order of the relevant genetic segment is C_{III} N C_I T_{II} Y C_{II} O P Q R[21, 22]), we tested the ability of λ phages containing mutations in C_I, C_{II}, C_{III}, or Y (λC_{17}) to direct the synthesis of exonuclease and endolysin. It is clear (Table 1, *C* and *D*) that each of these mutants, as well as λvir, is capable of directing the production of both enzymes in a normal manner.

Discussion.—The data presented in this paper question the validity of the assignment of the N cistron of λ as the structural gene for the λ-exonuclease. Although it is true that of all the mutants tested only those in N failed to produce exonuclease, it is also apparent that N mutants differ from the other early mutants O, P, and Q in that they show extremely low leakiness and they alone of this group fail to synthesize endolysin. Just as the inability of N mutants to synthesize endolysin does not prove that N is the structural gene for that enzyme, neither can their inability to form exonuclease be considered proof that N is the structural gene for this enzyme. It is possible that the absence of nuclease in N mutants is but one manifestation of the extreme defectiveness resulting from N lesions.

Several diverse observations, other than those presented here, attest to the deficiency of various λ-associated functions in N mutants. Brooks[2] had deduced from genetic experiments that N, O, and P mutants of λ fail to synthesize λ DNA. This deduction has been directly confirmed by Joyner *et al.*,[18] who assayed λ DNA by specific hybridization on DNA agar columns. The latter workers have also found that N mutants produce extremely little λ mRNA. Fuerst and Eisen[23] have observed that N mutants uniquely fail to undergo prophage autocuring. Finally, Lieb,[24] in her studies with temperature-inducible mutants, λC_I^{ts}, has observed that under appropriate conditions, cells containing the double mutant λC_I^{ts}, sus N fail to undergo the loss of viability at elevated temperature that is characteristic of thermoinducible lysogens.[25] We have demonstrated here that under our con-

ditions of treatment with mitomycin C, all of the N mutants tested lost their immunity to a homologous superinfecting phage. This immunity is conferred on the lysogenic cell by the prophage C_I repressor product.[4] Loss of immunity may be the direct result of repressor inactivation and thus the earliest event in lysogenic induction. It may also be the only event that can reproducibly be demonstrated in induced defective N lysogens.

We should like to propose an hypothesis for the function of the N cistron that seems to be consistent with the evidence that has been discussed. This hypothesis does not presuppose the nature of the specific protein produced by N and thus would seem to be reasonable whether or not this specific protein should prove to be the λ-exonuclease. The hypothesis involves the following assumptions:

(1) The N cistron is situated in that half of the λ DNA molecule which has long been inferred to be concerned with "early" functions.[27] Recently it has been shown that it is this half of the λ genome that is transcribed into mRNA early in λ infection[28] or lysogenic induction.[29]

(2) N cannot be considered to be an operator locus since it is defined as a complementation region.[1]

(3) Our data concerning N mutants cannot be explained on the basis of strong polarity effects. In support of this contention are the following: (*a*) The various N mutants used in these experiments span most of the N cistron,[26, 22] but we observed no gradation in the amount of endolysin produced; in all cases there was none. (*b*) More pertinent is the fact that on the basis of other studies both we[17] and Joyner *et al.*[18] have data strongly indicating that exonuclease and endolysin are encoded in different operons, in which case polarity could not be invoked.

(4) The synthesis of λ-exonuclease is in itself not sufficient to allow the synthesis of later enzymes, such as endolysin. This assumption is supported by our findings with mutant T_{11}.

(5) The inability of N mutants to synthesize endolysin is not due simply to inability to produce λ DNA. In support of this argument we note that Joyner *et al.*[18] have been unable to detect λ DNA synthesis with mutants N, O, and P. Furthermore, although we found that mutants O and P were more leaky with respect to phage formation than were N mutants, the amount of endolysin produced by O and P (10–20% of wild-type level) does not seem to correlate well with the degree of leakiness observed (with O, leakiness was about 5×10^{-4}; with P, about 10^{-2}).

We propose that N may be a regulatory cistron, the protein product of which "turns on" λ functions by allowing the initiation of transcription of early λ cistrons. Joyner *et al.*[18] have noted that Q mutants of λ, although they synthesize normal amounts of λ DNA, produce a reduced level of λ mRNA, endolysin, and tail antigens, and have suggested that the Q cistron is a control locus for late λ functions. In an analogous manner we suggest that the N cistron controls the transcription and subsequent expression of the early cistrons of λ.

Summary.—We have presented evidence derived from a study of the ability of various early mutants of λ to synthesize exonuclease and endolysin which indicates that the assignment of cistron N as the structural gene for the nuclease may be invalid. The available data are compatible with the hypothesis that the N cistron product is required for the initiation of transcription of that portion of the λ genome concerned with early functions.

We wish to express our gratitude to the several investigators mentioned in this paper who have permitted us to refer to their unpublished work.

[1] Campbell, A., *Virology*, **14**, 22 (1961).
[2] Brooks, K., Ph.D. thesis, University of Rochester (1964).
[3] Kaiser, A. D., *J. Mol. Biol.*, **4**, 275 (1962).
[4] Kaiser, A. D., and F. Jacob, *Virology*, **4**, 509 (1957).
[5] Isaacs, L. N., H. Echols, and W. S. Sly, *J. Mol. Biol.*, **13**, 963 (1965).
[6] Jacob, F., and C. R. Fuerst, *J. Gen. Microbiol.*, **18**, 518 (1958).
[7] Campbell, A., and A. del Campillo-Campbell, *J. Bacteriol.*, **85**, 1202 (1963).
[8] Radding, C. M., *Biochem. Biophys. Res. Commun.*, **15**, 8 (1964).
[9] Radding, C. M., these PROCEEDINGS, **52**, 965 (1964).
[10] Korn, D., and A. Weissbach, *J. Biol. Chem.*, **238**, 3390 (1963).
[11] Korn, D., and A. Weissbach, *Biochim. Biophys. Acta*, **61**, 775 (1962).
[12] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[13] Weissbach, A., and D. Korn, *J. Biol. Chem.*, **238**, 3383 (1963).
[14] Sekiguchi, M., and S. S. Cohen, *J. Mol. Biol.*, **8**, 638 (1964).
[15] Protass, J. J., and D. Korn, these PROCEEDINGS, **55**, 832 (1966).
[16] Korn, D., and A. Weissbach, *Virology*, **22**, 91 (1964).
[17] Protass, J. J., and D. Korn, in preparation.
[18] Joyner, A., L. N. Isaacs, W. F. Dove, W. S. Sly, and H. Echols, personal communication.
[19] Groman, N. B., and G. Suzuki, *J. Bacteriol.*, **84**, 596 (1962).
[20] Séchaud, J., and E. Kellenberger, *Ann. Inst. Pasteur*, **90**, 102 (1956).
[21] Fischer-Fantuzzi, L., and E. Calef, *Virology*, **23**, 209 (1964).
[22] Jacob, F., personal communication.
[23] Fuerst, C. R., and H. Eisen, personal communication.
[24] Lieb, M., personal communication.
[25] Sussman, R., and F. Jacob, *Compt. Rend.*, **254**, 1517 (1962).
[26] Naha, P. M., and A. Campbell, *Bacteriol. Proc.*, Abstract V37, 103 (1965).
[27] Jacob, F., and E. L. Wollman, *Sexuality and the Genetics of Bacteria* (New York: Academic Press, 1961), chap. 15.
[28] Skalka, A., *Carnegie Inst. Wash. Year Book*, **64**, 526 (1965).
[29] Naono, S., and F. Gros, *Compt. Rend.*, **261**, 3486 (1965).

---

*LOCATION OF GENETIC LOCI OF SOLUBLE RNA ON BACILLUS SUBTILIS CHROMOSOME**

BY M. OISHI, A. OISHI, AND N. SUEOKA

DEPARTMENT OF BIOLOGY, PRINCETON UNIVERSITY

Communicated by Paul Doty, March 28, 1966

Recently we have established a system in *Bacillus subtilis* in which gene replication proceeds synchronously from the origin of the chromosome using the germination stages of spores (Oishi, Yoshikawa, and Sueoka, 1964).[1] This system has made it possible to identify the replicated parts of the chromosome by density transfer experiments and was applied to decide and analyze the genetic loci corresponding to ribosomal RNA (16*S* and 23*S*) on the *B. subtilis* chromosome (Oishi and Sueoka, 1965).[2] Since little is known about the genetic background of soluble RNA in contrast to its biological importance, it is now quite interesting to identify