D. Doniach, *Clin. Exp. Immunol.* **45**, 316 (1981).
30. H. Rabin *et al.*, *J. Immunol.* **127**, 1852 (1981).
31. F. M. Brodsky, P. Parham, C. J. Barnstable, M. J. Crumpton, W. R. Bodmer, *Immunol. Rev.* **47**, 1 (1979).
32. I. Beckman, *Immunol. Today* **5**, 30 (1984).
33. We thank Mr. Russell and Professor Harrison for surgical specimens and access to their patients; J. R. Lamb, P. Delves, and P. Wolker for advice; P. C. L. Beverley, J. Bodmer, and W. Bodmer, M. Crumpton, V. Van Heyningen, J. Ledbetter, I. F. C. McKenzie, and T. Waldmann for monoclonal antibodies; Sandoz (Vienna) for generous gifts of recombinant IL-2; M. Contreras and her colleagues at National Blood Transfusion Centre, Edgware, for supplies of blood cells and serum; L. Hammond and J. Weingast Johnson for assistance; P. Wells for preparing the manuscript; I. M. Roitt and D. Doniach for interest in this project; M. C. Raff for comments on the manuscript; and the Juvenile Diabetes Foundation (United States), British Diabetic Association, Novo (Denmark), NIH, the Joint Research Board at St. Bartholomew's Hospital, Medical Research Council, and the Imperial Cancer Research Fund for financial support. M.L. is a fellow of the Juvenile Diabetes Foundation.

12 October 1984; accepted 9 January 1985

Genotoxicity of Formaldehyde in Cultured Human Bronchial Fibroblasts

Abstract. *Formaldehyde, a common environmental pollutant, inhibits repair of* O^6*-methylguanine and potentiates the mutagenicity of an alkylating agent,* N*-methyl-*N*-nitrosourea, in normal human fibroblasts. Because formaldehyde alone also causes mutations in human cells, the compound may cause genotoxicity by a dual mechanism of directly damaging DNA and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by other chemical and physical carcinogens.*

Formaldehyde is a ubiquitous environmental pollutant found in many occupational settings and in tobacco smoke, consumer products, and exhaust from gasoline and diesel combustion (*1*). The compound can also be generated by oxidative metabolism of many xenobiotics (*1*). For example, the carcinogen *N*-nitrosodimethylamine (DMNA) is metabolized to equimolar concentrations of methyldiazonium ion and formaldehyde. Formaldehyde causes single-strand breaks in DNA and DNA-protein cross-links; inhibits DNA excision repair in human cells (*2, 3*); is mutagenic in *Drosophila* larvae, bacteria, and fungi (*4*); is a respiratory carcinogen in rodents (*5*); and has been judged to pose a potential carcinogenic risk to humans (*1*).

The mutagenic and carcinogenic effects of *N*-nitrosamines, such as DMNA, and *N*-nitrosamides, such as *N*-methyl-*N*-nitrosourea (NMU), have been related to the degree of alkylation at the O^6-position of guanine in target tissue DNA and the ability of the tissue to remove this lesion (*6–9*). NMU methylates DNA at several sites, including O^6-guanine and *N*7-guanine (*10, 11*). The 7-methylguanine lesion is repaired slowly (half-life, 40 to 50 hours) (*11*), while O^6-methylguanine is repaired by O^6-alkylguanine DNA alkyltransferase at a much faster rate. Thus over short periods decreases in the ratio of O^6-methylguanine to 7-methylguanine can be used as a measure of O^6-methylguanine repair.

We investigated the repair of promutagenic O^6-methylguanine lesions induced by NMU in human bronchial fibroblasts. After a 1-hour exposure of these cells to 200 μM NMU, ratios of O^6-methylguanine to 7-methylguanine of between 0.029 and 0.054 were found (Table 1) (*12*). After 5 hours of posttreatment incubation of the cells in NMU-free medium, the ratio decreased from a mean of 0.041 to 0.009. Therefore only 20 percent of the O^6-methylguanine observed immediately after treatment with NMU for 1 hour remained in DNA 5 hours later. This observation is consistent with the kinetics of O^6-methylguanine and 7-methylguanine repair previously observed in human skin fibroblasts (*13*). However, when NMU-treated cells were incubated in the presence of 100 or 300 μM formaldehyde, a significantly lower rate of O^6-methylguanine removal was observed in three independent experiments. The removal of 7-methylguanine was not affected by formaldehyde and occurred at a rate (5 to 10 percent between hours 1 and 6) similar to that reported previously (*13*).

Formaldehyde could inhibit the repair of O^6-alkylguanine DNA alkyltransferase by several mechanisms, including steric hindrance of this DNA repair enzyme due to cross-links between DNA and DNA-associated proteins formed by formaldehyde. Because O^6-alkylguanine DNA alkyltransferase has a cysteine at its active site (*9*) and aldehydes readily bind to cysteine, formaldehyde and aldehydes formed by lipid peroxidation caused by formaldehyde-induced membrane damage could inhibit removal of O^6-methylguanine by binding to the active site of the alkyltransferase. This hypothesis is supported by recent observations that exposure of human bronchial epithelial and fibroblastic cells in vitro to formaldehyde or other thiol-binding aldehydes, such as acrolein and 4-hydroxy alkenals, preferentially inhibit O^6-alkylguanine DNA alkyltransferase activity when compared to that of another DNA repair enzyme, uracil DNA glycosylase (*14*).

To further study the pathobiological consequences of formaldehyde exposure and inhibition of O^6-methylguanine repair, we investigated the cytotoxic and mutagenic effects of formaldehyde and NMU separately and in combination. Cytotoxicity was determined by measuring the colony-forming efficiency of human fibroblasts that had been exposed to 100 to 800 μM NMU for 1 hour or 50 to 175 μM formaldehyde for 5 hours or to both as in the previous experiments, albeit at clonal rather than mass culture density. Compared to cells growing in logarithmic phase at clonal density, nearly confluent and slowly growing cultures

Table 1. Effect of formaldehyde on the removal of O^6-methylguanine from DNA of human fibroblasts exposed to NMU. Confluent human bronchial fibroblasts (HBF 357) were used to minimize the effects of cell cycle variations on growing cells. Cells were grown to confluence (15×10^6 to 16×10^6 cells per flask) in GDS medium (*18*). Sixteen flasks (four per determination) were incubated with tritiated NMU (1.6 Ci/mmol, New England Nuclear) at a concentration of 200 μM in 0.1 percent (by volume) dimethyl sulfoxide in phosphate-buffered saline (PBS; 4.4 ml per flask) for 1 hour at 37°C. After 1 hour the cells were washed once with PBS and then incubated in the presence or absence of formaldehyde [100 or 300 μM in 20 ml of EGM:LHC-1 (1:1) medium (*18*) per flask] for an additional 5 hours. At indicated times the cells were washed twice with PBS, harvested by trypsinization, and frozen as pellets at −70°C. DNA was purified by treatment with proteinase K and ribonuclease followed by phenol extraction and precipitation in ethanol by standard procedures. The levels of methylated DNA purines were analyzed by hydrolyzing the DNA in 0.1*M* HCl for 1 hour followed by separation by high-performance liquid chromatography (*19*). Values are ratios of O^6-methylguanine to 7-methylguanine.

Treatment	Experiment			Mean (percent)
	1	2	3	
NMU	0.054	0.029	0.039	0.041 (100)
NMU + fresh medium	0.008	0.006	0.012	0.009* (21)
NMU + 100 μM formaldehyde	0.014	0.014	0.015	0.014* (35)
NMU + 300 μM formaldehyde	0.024	0.019	0.019	0.021* (52)

*Each ratio is significantly different from other ratios ($P \leq 0.05$, Freidman test and Student's *t*-test).

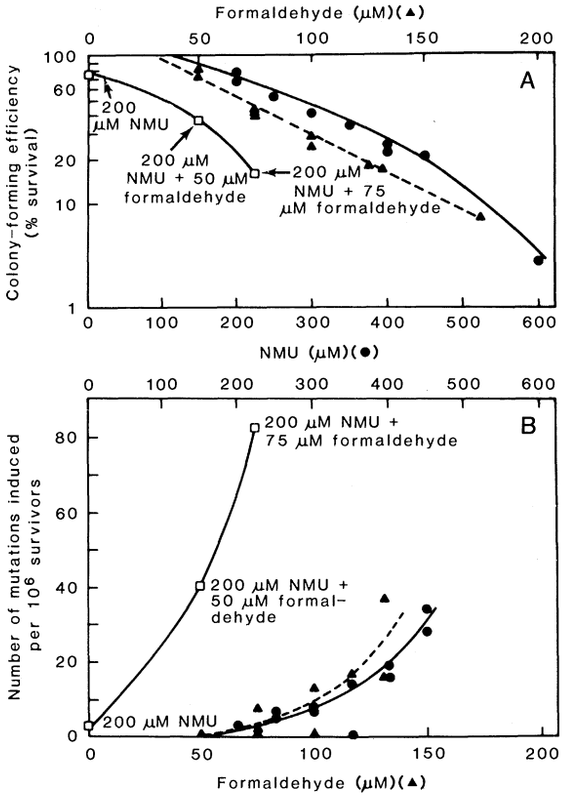

Fig. 1. Analysis of the cytotoxicity and mutagenicity resulting from treatment of human fibroblasts with NMU and formaldehyde (*20*). (A) Cytotoxicity of NMU alone (a single 1-hour exposure at the indicated concentration) and of formaldehyde alone (a 1-hour exposure at the indicated concentration followed by a second exposure at the same concentration for 4 hours). Also shown is the cytotoxicity resulting from exposure to 200 μ*M* NMU for 1 hour in the presence of 50 or 75 μ*M* formaldehyde followed by 4 hours of exposure to a fresh solution of formaldehyde at the same concentration. (B) Frequency of 6-thioguanine–resistant mutants induced as a consequence of treatment with NMU, formaldehyde, or a combination of the two.

of human fibroblasts repair cytotoxic damage and have substantially higher survival rates (*3*). The effect of low concentrations of formaldehyde or NMU or both was assessed by measuring the frequency of induction of 6-thioguanine–resistant mutants in human fibroblasts after their exposure to the compounds (Fig. 1). Although the exposure times differed, on a molar basis formaldehyde was three times more mutagenic than NMU (Fig. 1). As reported by others (*15, 16*), NMU and formaldehyde were each weak mutagens at the concentrations tested. However, addition of 50 or 75 μ*M* formaldehyde to 200 μ*M* NMU-treated cells resulted in a mutation frequency that was significantly greater than that found with either agent alone.

Formaldehyde may increase the mutagenicity of NMU by inhibiting O^6-methylguanine repair. This hypothesis is strengthened when the frequency of 6-thioguanine–resistant mutants is analyzed as a function of the logarithm of the percentage of survival (Fig. 2). The curves for NMU and for formaldehyde have similar magnitude and shape. However, a much steeper curve is found when formaldehyde and NMU are combined under conditions known to inhibit the repair of O^6-methylguanine. This indicates that the combination of NMU and formaldehyde has a higher mutagenic efficiency than does either agent alone. This is to be expected if the persistence of the O^6-methylguanine lesion has a more significant effect on induced mutation than it does on cell killing. Thus there is a good correlation between the inhibition of O^6-methylguanine removal and the synergistic increase in mutation frequency in the presence of formaldehyde. This is also further indirect evidence that O^6-methylguanine is a promutagenic DNA lesion. In addition, NMU may inhibit the repair of promutagenic lesions caused by formaldehyde.

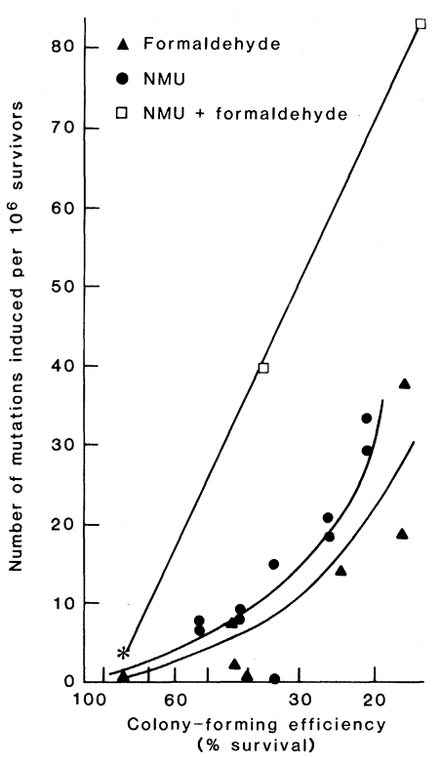

Fig. 2. Mutagenicity as a function of cytotoxicity of formaldehyde, NMU, or 200 μ*M* NMU combined with increasing amounts of formaldehyde (50 and 75 μ*M*). The asterisk indicates 200 μ*M* NMU alone.

The capability of cells to repair O^6-methylguanine in DNA is thought to be closely related to their sensitivity to *N*-nitroso compounds in terms of cytotoxicity, mutagenicity, and carcinogenicity (*6, 10, 13*). Because both alkyldiazonium ions and aldehydes are formed during metabolic activation of *N*-nitrosamines, we recently suggested that such aldehydes act in concert with the alkyldiazonium ions in causing pathological effects of *N*-nitrosamines (*17*). The results presented here are consistent with this hypothesis. Furthermore, we speculate that the enzyme systems responsible for metabolism of aldehydes are important in the susceptibility of organs to the carcinogenic effects of *N*-nitrosamines.

ROLAND C. GRAFSTROM*
Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20205
RODGER D. CURREN
LI L. YANG
Microbiological Associates, 5221 River Road, Bethesda, Maryland 20816
CURTIS C. HARRIS†
Laboratory of Human Carcinogenesis, National Cancer Institute

References and Notes

1. The Formaldehyde Panel, *Environ. Health Perspect.* **43**, 139 (1982).
2. R. C. Grafstrom, A. J. Fornace, Jr., H. Autrup, J. F. Lechner, C. C. Harris, *Science* **220**, 216 (1983).
3. R. C. Grafstrom, A. J. Fornace, Jr., C. C. Harris, *Cancer Res.* **44**, 4323 (1984).
4. C. Auerbach, M. Moutschen-Dahmen, J. Moutschen, *Mutat. Res.* **39**, 317 (1977).
5. J. A. Swenberg, W. D. Kerns, R. I. Mitchell, E. J. Gralla, K. L. Pavkov, *Cancer Res.* **40**, 3398 (1980).
6. P. N. Magee, R. Montesano, H. Preussman, *Am. Chem. Soc. Monogr.* **173**, 449 (1977).
7. A. Loveless, *Nature (London)* **223**, 206 (1969).
8. P. Kleihues and G. P. Margison, *J. Natl. Cancer Inst.* **53**, 1839 (1974).
9. A. E. Pegg, *Rev. Toxicol.* **5**, 83 (1983).
10. T. Lindahl, *Annu. Rev. Biochem.* **51**, 61 (1982).
11. B. Singer, *J. Natl. Cancer Inst.* **62**, 1329 (1979).
12. Since these ratios are comparatively low (the ratio of O^6-methylguanine to 7-methylguanine is approximately 0.14 when 200 μ*M* NMU is incubated for 1 hour with a nonrepairing Chinese hamster V79 cell line) [R. F. Newbold, W. Warren, A. S. C. Medcalf, J. Amos, *Nature (London)* **283**, 596 (1980)], a substantial amount of O^6-methylguanine repair occurred during the 1-hour exposure period.
13. A. S. C. Medcalf and P. D. Lawley, *Nature (London)* **289**, 796 (1981).
14. R. Grafstrom *et al.*, unpublished results.
15. A. S. C. Medcalf and M. H. Wade, *Carcinogenesis* **4**, 115 (1983).
16. V. S. Goldmacher and W. G. Thilly, *Mutat. Res.* **110**, 417 (1983).
17. C. C. Harris, R. C. Grafstrom, J. F. Lechner, H. Autrup, in *Banbury Report 12: Nitrosamines and Human Cancer*, P. N. Magee, Ed. (Cold

Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), p. 121.
18. J. F. Lechner, A. Haugen, I. A. McClendon, E. W. Pettis, *In Vitro* **18**, 633 (1982).
19. A. E. Pegg, L. Wiest, R. S. Foote, S. Mitra, W. Perry, *J. Biol. Chem.* **258**, 2327 (1983).
20. Cytotoxicity of NMU, formaldehyde, or a combination of the two was measured by plating 150 to 3000 human fibroblasts per 35-mm dish, allowing 12 hours for attachment, and introducing the chemicals. The cells were refed with fresh culture medium weekly until clones developed to macroscopic size, at which time they were fixed, stained, and counted. Duplicates of four dishes were used per experimental datum point. The cloning efficiencies for the solvent-treated controls in four separate experiments ranged from 33 to 47 percent. The frequencies of 6-thioguanine–resistant mutations induced by formaldehyde or NMU or both were calculated from data obtained in three independent experiments. The mutation assays were conducted as described by L. L. Yang, V. M. Maher, and J. J. McCormick [*Mutat. Res.* **94**, 435 (1982)]. Exponentially growing cells (1×10^6 to 5×10^6) were seeded into 150-cm^2 flasks at a density calculated to give 2.5×10^5 surviving cells per flask (a total of at least 10^6 surviving cells per treatment). A growth medium consisting of Eagle's minimum essential medium and Earle's salts supplemented with 10 percent fetal calf serum (Sterile Systems) and gentamicin was used throughout the experiments. The cells were exposed to the test compounds within 12 hours of seeding. Just before exposure of the cells, the growth medium was replaced with serum-free medium. NMU (dissolved in dimethyl sulfoxide) or formaldehyde (diluted in sterile distilled water) or the combination was introduced by micropipette (final concentration of dimethyl sulfoxide, <0.5 percent). For experiments with NMU alone, the carcinogen-containing medium was removed after 1 hour and fresh serum-free medium was added for an additional 4 hours. For experiments with the combination of NMU and formaldehyde, both chemicals were present for 1 hour before the treatment medium was replaced with fresh serum-free medium containing various concentrations of formaldehyde. Incubation continued for another 4 hours, at the end of which the formaldehyde-containing medium was replaced with fresh growth medium. For experiments with formaldehyde alone, the procedure was identical except that no NMU was present during the first hour of exposure. The surviving cells were allowed to undergo six to eight population doublings (7 to 9 days after treatment) in order to fully express the mutant phenotype. Population doublings were estimated from electronic counts of cells that had been seeded into small flasks at identical densities and given identical treatments to those described above. The cells were kept in rapid growth during the expression period by replating into new flasks as necessary. For selection of mutants, cells from three or more flasks were pooled and 1×10^6 to 2×10^6 cells were plated for selection in 40 μM 6-thioguanine–containing medium at a density of 500 cells per square centimeter in 100-mm dishes. At the same time cells were seeded at clonal densities (150 cells per 35-mm dish) in growth medium to determine the replating efficiency. Incubation under the selective or nonselective conditions was continued for 14 days with one refeeding. Qualitatively identical results were seen with shorter (4- to 6-day) mutation expression periods. Reconstruction experiments with Lesch-Nyhan cells deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) were conducted for each treatment condition at each selection day to determine the efficiency of recovery of mutant cells in the presence of cells positive for HGPRT. The efficiency of recovery in these experiments was 60 to 100 percent. Mutation frequency was calculated by determining the frequency of mutant clones per number of cells seeded and correcting for cloning efficiency and efficiency of recovery. The spontaneous mutation frequency did not exceed 10×10^{-6} mutants per survivor for the mutagenesis experiments.
21. We appreciate the technical aid of W. Pettis and the secretarial assistance of N. Paige. Supported in part by Swedish Cancer Society grant 1623-B-84-03R to R.C.G.
* Present address: Department of Toxicology, Karolinska Institutet, S-10401 Stockholm, Sweden.
† To whom correspondence and requests for reprints should be addressed.

17 May 1984; accepted 17 December 1984

Thermosensitivity of a DNA Recognition Site: Activity of a Truncated *nut*L Antiterminator of Coliphage Lambda

Abstract. *Antitermination is an important transcriptional control. In bacteriophage lambda, the presence of the* nut *antiterminators between the promoters and terminators results in relatively unhindered transcription when the lambda* N *gene product and necessary host factors are supplied. This antitermination system has been rendered thermosensitive by modification of the* nut *site. A fragment of* λ *DNA [74 base pairs (bp) in length] that contained the 17-bp* nut*L core sequence, but lacked the 8-bp* box*A sequence, was cloned in a* p_p-N-t_{L1}-galK *plasmid between the* p_p *promoter and gene* N. *This fragment mediated antitermination of transcription at 30°C, as measured by assaying* galK *gene expression in* Escherichia coli. *At 42°C, however, antitermination at the* λ t_{L1} *terminator was abolished. Antitermination at 42°C was restored by replacing the 74-bp* nut*L fragment with longer sequences containing both* nut*L and* box*A or by cloning a synthetic* box*A sequence ahead of the 74-bp* nut*L fragment. Thus, efficient antitermination required both* box*A and the 17-bp* nut*L core, with the latter becoming conditionally defective when the* box*A sequence was deleted.*

Termination and antitermination of transcription are examples of negative and positive regulatory mechanisms that control gene expression. Terminator signals block the progress of transcription, while special recognition sites, such as *nut* (*1–5*), mark those operons that are subject to antitermination. In bacteriophage λ the *nut* sequences, which are located downstream of the promoters p_L and p_R and upstream of a series of terminators in the phage genome, are the recognition sites for the *N*-dependent transcriptional antitermination function. The *nut*L site (Fig. 1A) (*1*) functions as an antitermination site not only in λ but

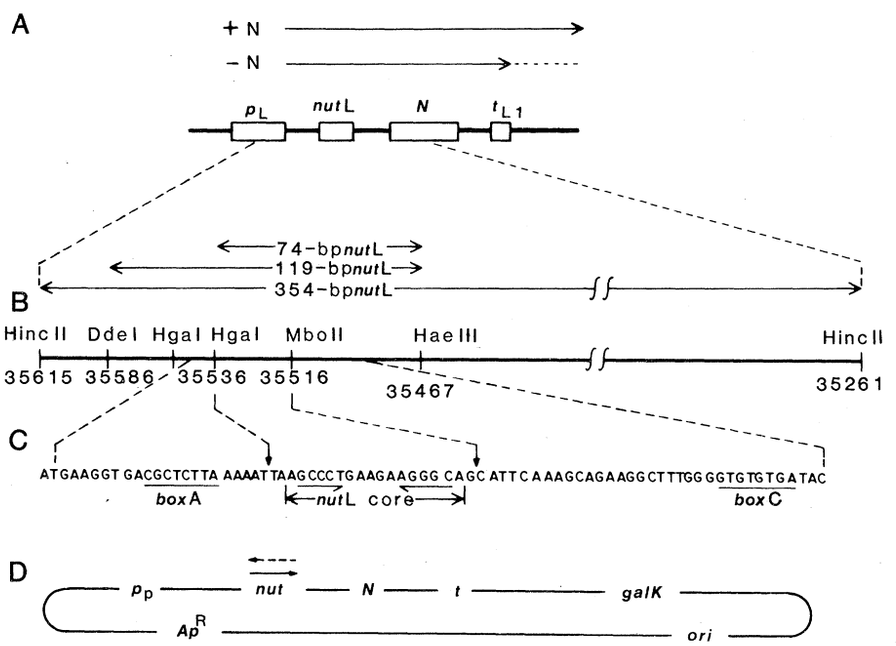

Fig. 1. Genetic and physical maps of the control regions of the leftward phage λ operon. The orientation of the map is opposite to that of the conventional λ map (*9*). (A) Diagrammatic representation of the p_L promoter, *nut*L antiterminator, gene *N*, and t_{L1} terminator in phage λ, with transcription originating at p_L and terminating at t_{L1} (lower arrow; $-N$). In the presence of gene *N* product, in conjunction with the *nut*L antiterminator, transcription proceeds across t_{L1} (upper arrow; $+N$). (B) Restriction map of the *nut*L region of λ. The locations of three λ DNA fragments are shown: (i) the 74-bp Hga I–Hae III fragment (*2*) containing the 17-bp *nut*L core (*10*) flanked by 7 bp upstream and 50 bp downstream, including the *box*C sequence (*4*); (ii) the 119-bp Dde I–Hae III fragment, containing the 17-bp *nut*L core flanked by 57 bp upstream [including the *box*A sequence (*4*)] and 50 bp downstream, including *box*C; (iii) the 354-bp Hinc II–Hinc II fragment. The coordinates of the restriction sites are specified (*9*). (C) Nucleotide sequence of the *nut*L region, including the 8-bp *box*A, the 17-bp *nut*L core, and the 8-bp *box*C. Nucleotide numbers (35536 and 35515) refer to the bases to the left of the restriction cut (*9*). (D) Schematic representation of plasmids pDX1, pDE3 (*2*), and p74nutL-1 derived from pKO3 (*11*), which carry the 74-bp *nut*L sequence. The *nut*L site is shown in its λ-like orientation (solid arrow) and in the reverse orientation (dashed arrow).