Ribonuclease P, the M1 RNA–C5 protein complex, cleaves not only precursors to tRNA's but also another substrate, the precursor to 4.5*S* RNA, a small stable RNA found in *E. coli* (*9*). The pM1 RNA–C5 protein complex can also effect this reaction. The arrow in Fig. 1, lane 3, points to the upper part of a doublet band in Fig. 1, lane 4. We have shown, using material isolated in preparative cleavage experiments, that the upper band in Fig. 1, lane 4, corresponds to mature 4.5*S* RNA and that it has the expected oligonucleotide, pGGGGC, at its 5′ terminus. Fingerprints of the cleavage products generated by pM1 RNA from both of our substrates are identical to those obtained when M1 RNA was used as the source of ribonuclease P activity [figure 7 in (*3*)].

The pM1 RNA used in our experiments was prepared by transcription in vitro and extracted with phenol prior to assay for nuclease activity. We earlier showed (*3*) that mature M1 RNA preparations, treated in the same way, lacked detectable protein. In the case of pM1 RNA, the only possible source of contaminating protein in the preparation is the transcription mixture used to synthesize the RNA. This mixture has no nuclease activity when incubated with the substrates we used. Thus, the ability of pM1 RNA preparations to cleave the precursor to $tRNA^{Tyr}$ and generate the 5′ terminus of the mature tRNA lies in the pM1 RNA itself. Furthermore, the molar concentration of product generated in reactions such as those illustrated in Fig. 1, lane 2, is greater than the concentration of pM1 RNA in the reaction mixture (*10*). In preparative reactions, pM1 RNA can generate a more than tenfold molar excess of product compared to its own concentration. pM1 RNA is unchanged in size during the course of the reaction (data not shown). These data indicate that pM1 acts catalytically, as is the case for M1 RNA (*3*).

Our results confirm and extend our previous findings (*3*) that an RNA is capable of acting as a catalyst. It has also been shown that a precursor to ribosomal RNA in *Tetrahymena* has the ability to carry out enzymatic reactions (*11*). In this report, we have shown that the precursor to an RNA species has catalytic capability and that this precursor, as well as the processed, mature molecule, interacts with a protein cofactor to make an enzymatic complex with a higher rate of reaction and wider substrate range than that shown by the precursor RNA alone. Until studies of the kinetic characteristics of these reactions provide more information, the only function we can ascribe to the extra sequences in pM1 RNA is that of a signal for the termination of transcription (*4*).

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10. Preparation of the precursor to *E. coli* $tRNA^{Tyr}$ and estimates of its concentration from specific radioactivity measurements were carried out as described (*8*). The reaction products in the experiments shown in Fig. 1 were analyzed on a 10 percent polyacrylamide gel in 89 m*M* tris-borate, 2.5 m*M* EDTA buffer. After autoradiography, the bands corresponding to intact precursor, processed tRNA and 5′ fragment were excised from the gel, and the radioactivity in each was determined by counting Cerenkov radiation. In preparative cleavage reactions with 18 fmole of pM1 RNA as enzyme and 6900 fmole of pTyr as substrate, 780 fmole of $tRNA^{Tyr}$ cleavage product are produced. In the experiment shown in Fig. 1, lane 2, at least 22 fmole of $tRNA^{Tyr}$ cleavage product results from the reaction catalyzed by 7.5 fmole of pM1 RNA.
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12. Plasmid pRR3 was digested with Eco RI, and 0.2 pmole of the 2-kb fragment that contains the gene for M1 RNA (provided by Dr. M. Baer) was used as template for transcription experiments in vitro (*4*). Reactions were carried out in 20 μl with the specific radioactivity of guanosine triphosphate adjusted to 12.5 μCi per 200 μ*M*.
13. Buffer A: 30 m*M* tris-HCl, *p*H 7.0, 50 m*M* NH_4Cl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* 2-mercaptoethanol. Buffer B: 50 m*M* tris-HCl, *p*H 7.6, 60 m*M* NH_4Cl, 10 m*M* $MgCl_2$. Buffer C: 50 m*M* tris-HCl, *p*H 7.6, 100 m*M* NH_4Cl, 60 m*M* $MgCl_2$, 5 percent glycerol.
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Molecular Dissection of Transcriptional Control Elements Within the Long Terminal Repeat of the Retrovirus

Abstract. *The retroviral long terminal repeat (LTR) contains transcriptional control elements that affect viral gene expression. By deletion mutagenesis of the genome of the cloned Abelson murine leukemia virus, regulatory signals could be mapped to at least three domains within the LTR. A defective 5′ LTR that did not sustain transforming gene function was complemented by an intact LTR positioned at the 3′ end of the genome. This versatility of the retroviral genome with respect to its transcriptional control elements appears to provide a strong selective advantage for viral gene expression.*

The proviral genome of a retrovirus contains characteristic directly repeated sequences designated long terminal repeats (LTR's). The LTR is synthesized from viral genomic RNA components specific to the 5′ (U5), 3′ (U3), and repeated terminus (R) regions of the molecule during reverse transcription into a U3-R-U5 configuration [for review see (*1*)]. Because of their location within the viral genome, as well as their resemblance to transposable elements, retroviral LTR's have been said to function in processes including virus transcription, integration, replication, and genome rearrangement (*2, 3*).

With respect to viral RNA transcription, certain LTR functions have been tentatively assigned. Nucleotide sequence analysis of LTR's derived from a number of avian and mammalian retroviruses has revealed the existence of consensus promoter sequences in the U3 region located at similar distances from the RNA initiation site in the R region (*2, 3*). Moreover, evidence from eukaryotic cell-free transcription systems has indicated that viral RNA transcripts initiate within the LTR (*4*). Evidence is also accumulating for the existence of sequences with an "enhancer"-like function within the U3 region of the LTR [for review see (*5*)]. In other systems, enhancer elements, which can act at a distance from the RNA start site and independent of orientation, appear to play an essential role in efficient gene function (*6*).

Abelson murine leukemia virus, a prototypical acute transforming retrovirus,

arose by recombination of Moloney murine leukemia virus (Moloney-MuLV) with a cellular *onc* sequence designated *abl*. Moreover, *abl* is translated from a message initiated within the 5′ LTR (*7*). Molecularly cloned A-MuLV demonstrates high-titered transforming activity as determined by transfection analysis on NIH/3T3 cells (*8*). Since A-MuLV transforming activity is dependent on efficient *abl* gene expression, we undertook deletion mutagenesis of the A-MuLV 5′ LTR in an effort to precisely locate its transcriptional control elements. We have also explored the possibility that the 5′ and 3′ LTR's can act in a cooperative manner to allow transforming gene function.

We used an A-MuLV deletion mutant, pHind, which lacks the distal 1.019 kilobases (kb) of the viral genome, including the 3′ LTR (*9*). This mutant transforms NIH/3T3 cells at a titer comparable to that of the wild-type A-MuLV genome (*10*). By use of pHind, any influence of the 3′ LTR on *abl* expression could be eliminated. In order to construct deletion mutants in TATA, CAAT (A, adenine; C, cytosine; and T, thymine), and the 5′ cap site, we cleaved pHind with the restriction enzyme Xba I and treated the linearized plasmid with Bal31 exonuclease. After preliminary screening with appropriate restriction enzymes, we established the precise extent of each deletion by DNA sequencing (*11*).

An A-MuLV mutant (pAva), in which the TATA box and the 5′ cap site were specifically deleted, showed no impairment in transforming activity (Fig. 1). Deletion of the CAAT box as well (pHX25) reduced A-MuLV focus formation by a factor of about 10. Finally, mutants pHX37 and pHX90, in which larger portions were deleted, including the polyadenylation (poly A) signal and other downstream sequences, lacked any detectable biologic activity. These findings indicate that the TATA box and the 5′ cap site are not essential for the expression of the *abl* gene. In the absence of the TATA box and the cap site, adjacent sequences seem to function as alternative promoter or initiation sites for transcription. Such sequences appear to be farther downstream since mutants with large deletions encompassing this downstream region—for example, pHX37 and pHX90—lacked transforming activity.

Nucleotide sequence analysis has revealed a 72– to 74–base pair (bp) repeat sequence in the U3 region of the LTR's of a number of retroviruses, including A-MuLV (*2, 3, 9*). A 72-bp unit has been identified within the genomes of papovaviruses (*5*). Deletion of this element in SV40 has been reported to reduce expression of the T antigen by a factor of at least 100 and to abolish virus viability (*6, 12*). Evidence that the retroviral 72-bp repeat can replace the SV40 repeat and restore SV40 viability has suggested that the retroviral 72-bp repeat may similarly play a role in efficient retroviral gene expression (*13*).

To explore the effect of this region of the LTR on A-MuLV transforming gene function, we constructed another series of LTR deletion mutants lacking one or both 74-bp repeats. The strategy used to generate these mutants is summarized in Fig. 2. A subgenomic plasmid, pMEP, which contained 1.8 kilobase pairs (kbp) of flanking cellular sequences upstream from the LTR, the 5′ LTR, and an additional 280 bp of viral sequences, was subjected to restriction with Pvu II alone or in combination with Xba I. This resulted in the generation of the mutants pP, lacking a single 74-bp repeat, and pPX, lacking both 74-bp repeats, along with 31 bp downstream from the repeats (Fig. 2). As shown in Fig. 1, loss of one 74-bp repeat did not impair A-MuLV transforming activity. However, loss of both repeats completely abolished this function.

Because the retrovirus genome is organized with an LTR at each terminus, we explored the possible cooperative effects of an intact 3′ LTR on A-MuLV deletion mutants lacking transforming activity as a result of a defective 5′ LTR. Thus, pHX37 and pPX deletion mutants

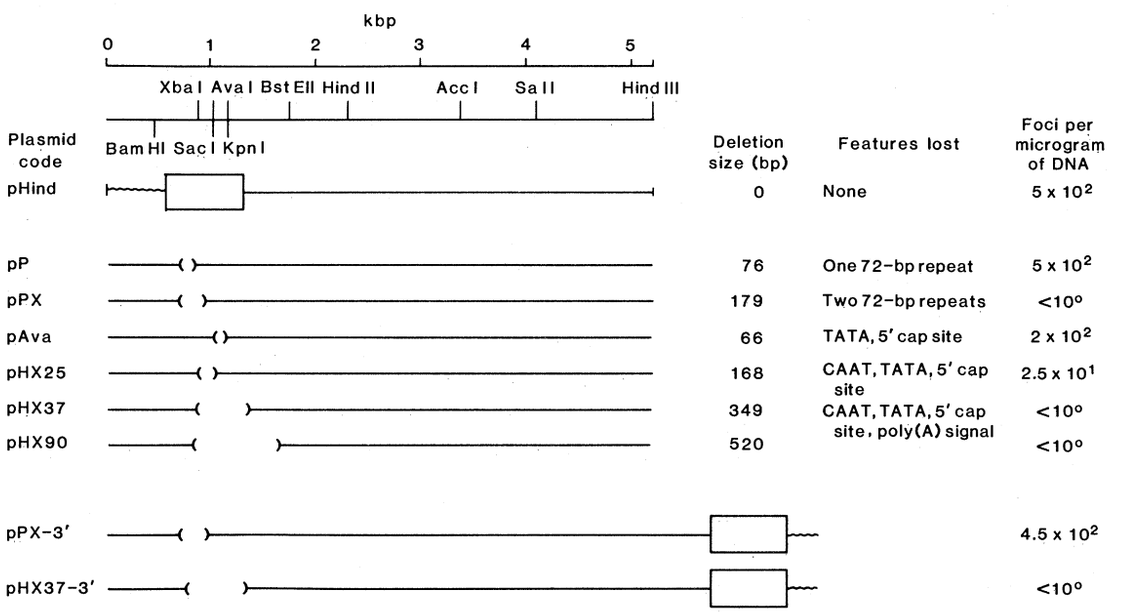

Fig. 1. Physical map of A-MuLV in the pHind recombinant plasmid (*10*) and schematic representation of the deletions present in the mutants analyzed in this study. A-MuLV sequences are represented by a solid line, an LTR is indicated as a rectangle, and flanking mink cellular sequences are marked by wavy lines. Details of the isolation of mutants pP, pPX, and pAva are given in the legend to Fig. 2. Mutant plasmids pHX25, pHX37, and pHX90 were isolated with the use of the pHind plasmid; pHind was linearized with restriction enzyme Xba I and then treated with Bal31 nuclease to produce progressive deletions covering sequences both upstream and downstream from the putative cap site. Mutants were first characterized by restriction enzyme mapping, and the extent of the deletion was further established by DNA sequencing (*11*). Plamid pPX-3′ with defective 5′ LTR and wild-type 3′ LTR was generated as follows. After pPX (5 μg) was digested with Hind III, the defective 5′ LTR–containing fragment was purified on a 0.7 percent low-melting agarose gel. The purified fragment was ligated to a 1.25-kbp fragment containing 3′ LTR and flanking mink cellular sequences, and the resulting DNA was cloned in pBR322 cleaved with Hind III and Eco RI. Plasmid pHX37-3′ was constructed with essentially the same method. The presence of a defective 5′ LTR and wild-type 3′ LTR in the recombinant plasmid was confirmed by restriction enzyme mapping. The sequences deleted in different mutant constructs are indicated by parentheses.

were cleaved with Hind III, and a wild-type 3′ LTR was inserted at this site (Fig. 1). The resulting recombinant plasmids, pHX37-3′ and pPX-3′, respectively, were analyzed for transforming activity in the NIH/3T3 transfection assay. As shown in Fig. 1, the addition of a 3′ LTR failed to complement the defective 5′ LTR in pHX37-3′. In contrast, the transforming activity of pPX-3′ was restored almost to wild-type levels.

Eukaryotic transcriptional units, which are recognized by RNA polymerase II, appear to contain at least two essential regions, designated promoters and enhancers (*5, 6*). The promoter region harbors the canonical TATA box which is thought to position RNA polymerase II near the site of transcription initiation (*12, 14*). Our studies demonstrate that retroviruses, like certain eukaryotic genes (*5, 6, 12*), do not require the TATA box for efficient gene expression. This is in contrast to prokaryotic genes in which even single base alterations in the promoter region can abolish transcriptional activity (*6*).

Structural analysis of a number of genes has established that the CAAT box in eukaryotic genes is located in a region 60 to 80 bp upstream from the RNA start site. The well-conserved nature of this signal implies that it functions as part of a larger transcriptional unit (*5, 15*). We have now found that loss of this signal in addition to the TATA box results in some reduction in *abl* transforming activity. Thus, our findings support the concept that these two signals allow more efficient gene expression when they act in concert.

Recent studies in several systems, including papovaviruses and some eukaryotic genes (*5, 6*), have identified enhancer elements upstream from the promoter site. These sequences are indispensable for the function of such genes in vivo (*5, 6*). In Moloney-MuLV, a region of 300 bp in the LTR that encompasses the 72-bp repeat has this function (*16*). Our studies map this activity to a region including only a single 74-bp repeat and no more than 31 bp of adjacent downstream sequences. An important feature of enhancer elements appears to be their ability to act at a distance, and independent of orientation, to facilitate gene expression (*6*). Our demonstration that the intact 3′ LTR can act at a distance of more than 5 kbp to restore biologic activity to a mutant lacking these sequences firmly establishes that this region in U3 has enhancer activity.

It has been suggested that enhancer elements act to provide a DNA entry site for RNA polymerase II or a polymerase-associated molecule (*5, 6*). Such a hypothesis would be consistent with the position and orientation independence of enhancement observed in our studies and those of others (*6*). Alternatively, the enhancer may alter chromatin structure or superhelicity to create regions of increased trancriptional activity (*5, 6*).

The present studies assign transcriptional regulation signals to at least three domains within the LTR; the first contains the CAAT box, the second contains

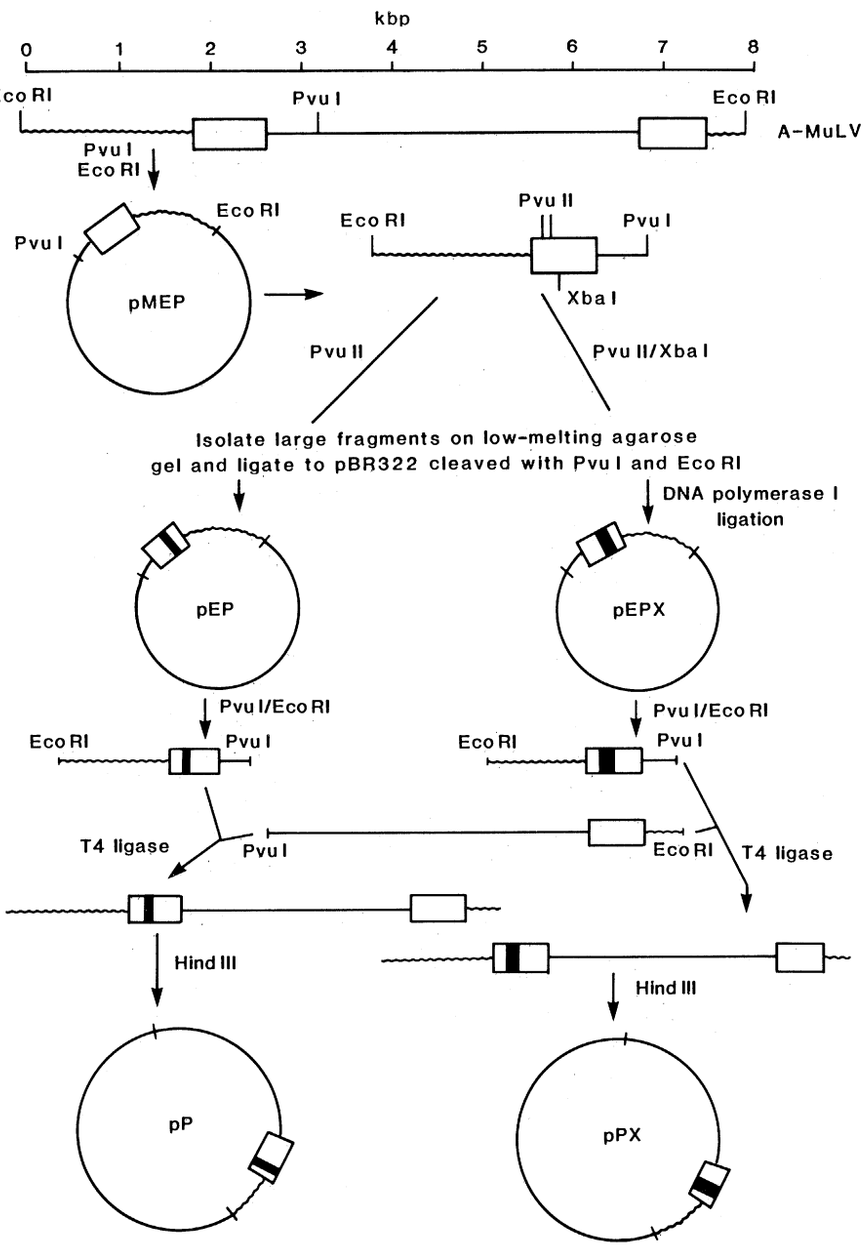

Fig. 2. Construction of A-MuLV mutants lacking one or both 74-bp repeats. In preparing plasmids pP and pPX, a 7.8-kbp insert containing A-MuLV proviral DNA (10 μg) (*8*) was digested with restriction enzyme Pvu I, and the resulting 2.7-kbp (containing 5′ LTR) and 5.1-kbp (containing the rest of the viral genome) fragments were separated on an 0.8 percent low-melting agarose gel. DNA was extracted and purified on a DEAE 52 column (*11*). The 2.7-kbp fragment was ligated to pBR322 DNA restricted with both enzymes Eco RI and Pvu I, and the ligated DNA was used to transform *Escherichia coli* cells C600 (*17*) to obtain recombinant plasmid pMEP. The 2.7-kbp fragment released from this plasmid was digested with Pvu II alone or with Pvu II and Xba I together. The resulting larger fragments were purified from the middle smaller fragment and ligated to pBR322 DNA cleaved with Pvu I and Eco RI. After ligation of pBR322 DNA to DNA fragments obtained by Xba I and Pvu II cleavage, the noncompatible ends were blunt-ended with DNA polymerase I and religated. Transformation was carried out as described above. From the tetracycline-resistant colonies, mutants with a truncated LTR were isolated. Those with deletions including 74-bp repeats were removed from the recombinant plasmid and ligated back to the 5.1-kbp viral fragment, and the resulting insert was cloned in pBR322 restricted with Eco RI. This plasmid was then cleaved with Hind III and subcloned at the Hind III site of pBR322. Mutant pAva was constructed with essentially the same strategy.

the TATA box and downstream sequences including RNA initiation sites, and the third contains the enhancer element. Our evidence suggests that the LTR is capable of utilizing alternative promoter and/or initiator signals when normal promoter signals are deleted or mutated. While an enhancer element seems to be an absolute necessity for viral gene function, the organization of the retrovirus genome allows the intact 3′ LTR to complement loss of the enhancer element in the 5′ LTR. This versatility of the virus genome with respect to its transcriptional control elements may provide the virus with a strong selective advantage and help to account for its ability to maintain an intimate association with the vertebrate genome.

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Fluorescence-Line-Narrowed Spectra of Polycyclic Aromatic Carcinogen–DNA Adducts

Abstract. *The laser excited fluorescence-line-narrowed spectrum of DNA modified with (±)-r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo*[a]*pyrene (BPDE), the ultimate carcinogenic metabolite of benzo*[a]*pyrene (BP), has been obtained in a water-glycerol-ethanol glass at 4.2 K. The spectrum was well resolved and highly characteristic of the chromophore. Comparisons were made between the spectrum of this modified DNA and the isolated deoxyguanosine-BPDE adduct and a series of other 7,8,9,10-tetrahydro-BP (THBP) derivatives. 9-Hydroxy-BP 4,5-oxide, which is also involved in the binding of BP to DNA, and THBP have very similar conventional broadband fluorescence spectra. However, the fluorescence-line-narrowed spectra of their derivatives were readily distinguishable either as individual components or as mixtures.*

Many carcinogens, including polycyclic aromatic hydrocarbons (PAH's), require metabolic activation to derivatives that bind covalently to DNA before they exert their carcinogenic effects. Conventional fluorescence spectra of DNA containing such derivatives, obtained at temperatures substantially above 4.2 K and with broadband excitation sources, have shed light on this activation process (*1*). Such spectra have fluorescence line widths of a few hundred reciprocal centimeters, and therefore species with similar structures cannot be distinguished in mixtures. This limitation can be circumvented with the use of fluorescence-line-narrowed (FLN) spectrometry (*2*, *3*), which affords line widths of a few reciprocal centimeters. The direct determination of substitutional PAH isomers in samples, such as solvent-refined coal, has been possible with the use of FLN spectrometry (*4*). This technique would be very valuable in the study of DNA adduct formation resulting from human exposure to PAH mixtures such as diesel engine emissions, cigarette smoke, or coke-oven emissions if FLN spectra could be obtained on DNA samples isolated from cells from such exposed individuals.

As a first step toward this goal, we

Table 1. The FLN spectra of THBP derivatives; λ_{exc}, excitation wavelength. Vacuum-corrected vibrational frequencies (in reciprocal centimeters) were measured relative to the fluorescence origin. Values are accurate to ± 10 cm^{-1}. The following chemicals, THBP (R7–10 = H in Fig. 1), (±)-7-hydroxy-THBP, 10-oxo-THBP, and 9-methoxy-BP 4,5-dihydrodiol, were supplied by R. G. Harvey, University of Chicago. The (±)-10-hydroxy-THBP was obtained by sodium borohydride reduction of the corresponding ketone. The BPDE was obtained through the Chemical Repository of the National Cancer Institute and was used to prepare DNA modified with BPDE (*11*). A side product of the modification reaction was the (±)-BP tetrol (7,8,9,10-tetrahydroxy-THBP). The BPDE-dG (R7–9 = hydroxy, R10 = deoxyguanosine attached through its 2-amino group in Fig. 1) was prepared by enzymic hydrolysis of the modified DNA (*13*). We purified all the derivatives, except the modified DNA, by reverse-phase HPLC, using mixtures of water and methanol. Letters in parentheses, *s*, m, and w, indicate strong (> 0.5), medium (0.5 to 0.2), and weak (< 0.2) peaks, respectively, relative to the most intense. Strengths are assigned qualitatively relative to the most intense vibrational frequency in the spectrum. The frequencies of many of the weak peaks have not been tabulated.

THBP (λ_{exc} = 3784 Å, 156 μ*M*)	7-Hydroxy-THBP (λ_{exc} = 3800 Å, 132 μ*M*)	10-Hydroxy-THBP (λ_{exc} = 3784 Å, 660 μ*M*)	Tetrol (λ_{exc} = 3771 Å, 220 μ*M*)	BPDE-dG (λ_{exc} = 3771 Å, 0.17 μ*M*)	BPDE-DNA (λ_{exc} = 3784 Å, 8 μ*M*)*
284(w), 311(w)	294(w)	270(w)	251(w)	251(w)	242(w)
331(w), 372(w)		467(w)	463(w)	456(w)	445(w)
460(w), 494(w)			551(w)	544(w)	552(w)
574(m)	582(w)	574(w)	592(w)	579(w)	592(w)
636(m)	635(w)	628(w)			
753(m)	759(w)	747(w)	785(m)	785(m)	770(m)
			864(m)	844(m)	836(w)
1072(w)	1069(w)	1072(w)	1073(w)	1069(w)	
1104(m)	1107(m)	1104(m)	1105(m)	1105(m)	1101(m)
1162(w)	1177(w)	1168(w)			
1245(s)	*1240(s)*	*1238(s)*	*1234(s)*	*1234(s)*	*1241(s)*
1270(s)	*1265(s)*	*1264(s)*	1278(m)	1278(m)	1272(m)
1333(w)	1321(w)	1320(w)	1310(w)	1317(w)	
1404(s)	*1408(s)*	*1420(s)*	*1411(s)*	*1404(s)*	*1404(s)*
1551(w)	1550(m)	1545(w)	1547(m)	1547(m)	1546(m)
1595(w)	1581(w)	1558(w)	1587(w)	1580(w)	1583(w)
1625(w)	1624(w)	1619(w)	1630(w)	1624(w)	1602(w)

*With respect to the adduct concentration, 0.4 percent of the bases were modified.