Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3778–3782, May 1990
Neurobiology

NICER elements: A family of nerve growth factor-inducible cAMP-extinguishable retrovirus-like elements

(PC-12 cells/long terminal repeat/Rasheed rat sarcoma virus/retrotransposon)

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Communicated by Arthur B. Pardee, January 25, 1990 (received for review October 28, 1989)

ABSTRACT **We have shown previously that the transcription of the gene designated *d5* is induced by nerve growth factor (NGF) in rat adrenal pheochromocytoma PC-12 cells and that this NGF induction is repressed by cAMP. In this paper we demonstrate that *d5* is a member of a gene family that contains several hundred members, which is closely related to retroviruses and retrotransposons, as demonstrated by the following observations: (*i*) the original d5 cDNA hybridized to numerous restriction fragments in genomic DNA; (*ii*) d5 cDNA hybridized to genomic clones with various intensities, and genomic clones can be isolated with a frequency suggesting that this family includes several hundred members; and (*iii*) there were minor sequence variations in four independently isolated cDNA clones that were homologous to d5 cDNA. Primer extension studies show that initiation of the 5.7-kilobase d5 mRNA(s) occurs at a unique site relative to a synthetic primer. The 5′ end of the cDNA sequence was homologous to Rasheed rat sarcoma virus; and a genomic clone contained several elements that are typical of a long terminal repeat (LTR), including a CCAAT box, a TATA box, a primer binding site, a poly(A) addition signal, and a poly(A) addition site. Furthermore, there is a LTR at the 3′ end of at least one of the genes in this family, and there appeared to be a four-base duplication at the probable site of integration into host DNA. Since several members of this family retain responses to NGF and cAMP, we conclude that the regulatory elements present in the LTR have been conserved in many members of this family. We have named this family of genes the NICER elements because they are a family of NGF-inducible cAMP-extinguishable retrovirus-like elements.**

Retroviruses have been extensively studied because of their roles in oncogenesis and other diseases, most notably AIDS. By the nature of their life cycle, retroviruses can integrate into the genome of infected germ-line cells and become a stable part of the host genome. Evidence for infectious and endogenous retroviruses has been found in all eukaryotes where they have been sought, including mouse, hamster, monkey, and human (for reviews, see refs. 1 and 2). Such endogenous viruses can affect cellular genes either by disruption (3, 4) or by regulating the expression of neighboring genes (5–8). To maintain a constant number of integrated viruses, the replication and integration of the retrovirus families must be under stringent regulation, because an unrestricted proliferation and transposition of these elements would be destructive to the host.

Many of the regulatory features of the retrovirus, including promoters and enhancers, are concentrated in the long terminal repeat (LTR; see ref. 2 for a review). Some LTRs contain regulatory elements that respond to the signal transduction systems present in the cell. For example, mouse mammary tumor virus and Moloney murine sarcoma virus contain regulatory elements that respond to glucocorticoids; and many retroviruses, including human immunodeficiency virus type 1 (HIV-1), formerly called HTLV-III, and avian reticuloendotheliosis virus type 2, contain sequences homologous to the glucocorticoid-responsive elements (9–11). It is reasonable to suggest that retroviruses have evolved their regulatory elements to respond appropriately to various cellular signals during their life cycle.

The d5 cDNA clone that originally was isolated as an NGF-inducible gene in rat adrenal pheochromocytoma PC-12 cells is induced not only by NGF but also by epidermal growth factor, fibroblast growth factor, and phorbol 12-myristate 13-acetate (12). The transcription of d5 is observed as early as 5 min but returns to basal levels by 3 hr; and its induction by NGF is repressed by cAMP (12). In this paper we show that the *d5* gene defines a family of endogenous rat retrovirus-like elements on the basis of several lines of evidence.‡ First, the 5′ end of the d5 cDNA clone has extensive homology to the LTR of Rasheed rat sarcoma virus (13). Second, this 565-base-pair (bp) LTR is repeated at both the 5′ and the 3′ end of at least one of the *d5* genomic clones. Third, this LTR contains several features of a eukaryotic promoter, including a TATA box, a CCAAT box, and a serum-responsive element (SRE). Fourth, this element also contains two sites that are essential for viral replication—a primer binding site and a polypurine tract. Finally, there are multiple copies of the element in the genomic DNA. Thus, we conclude that we have identified a family of retrovirus-like elements whose transcription is induced by growth factors.

MATERIALS AND METHODS

Additional d5 cDNA clones were obtained by screening the cDNA library that had been constructed previously (12) with the pUC19 plasmid containing the original d5 cDNA, labeled by the random hexamer priming method (14), as a probe (Fig. 1). To obtain restriction patterns of these clones, phage DNAs were digested with *Eco*RI, *Bam*HI, or *Hin*dIII or with combinations of these enzymes and were fractionated on a 1% agarose gel. The rat liver genomic library used in this study (15) was screened with three different probes: (*i*) a synthetic 20-mer that was chemically synthesized based on the sequence information of the 5′ end of cDNA clones (d5-23, d5, and d5-28 in Fig. 1), (*ii*) a plasmid subclone containing the original d5 insert, and (*iii*) a plasmid containing a 400-bp *Eco*RI fragment from the 3′ end of the cDNA clone of d5-32 (Fig. 1). Genomic clones that hybridized to all three

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Abbreviations: NGF, nerve growth factor; NICER elements, NGF-inducible cAMP-extinguishable retrovirus-like elements; LTR, long terminal repeat; SRE, serum-responsive element.

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M33534 and M33535).

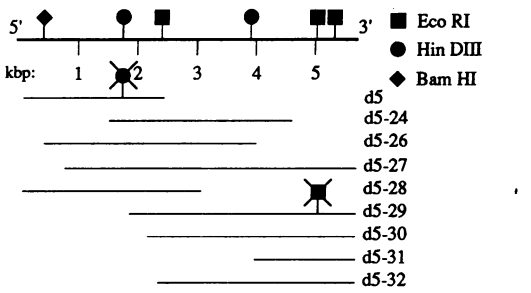

FIG. 1. Restriction map of cDNA clones of *d5*. The *Eco*RI, *Bam*HI, and *Hin*dIII sites are shown. *Eco*RI sites at both ends of each cDNA have been generated during cDNA synthesis. The d5-28 clone contained two *Eco*RI fragments [1.2 and 0.5 kilobases (kb)] that mapped to the 3' end of the cDNA clone but were not related to any other parts of d5.

probes were picked for further analysis. For Southern analysis, PC-12 DNA (20 μg) was digested with various restriction enzymes and size-fractionated in a 1% agarose gel. A Southern blot of DNA isolated from rat, mouse, human, quail, *Xenopus*, and *Drosophila* was kindly provided by Hyo Sung Ro of the Dana–Farber Cancer Institute. Hybridization was carried out at 42°C in 50% formamide/5× Denhardt's solution/5× SSPE/0.1% sodium dodecyl sulfate (SDS)/10 μg of salmon sperm DNA per ml (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA; Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin). The blots were washed at 68°C in 0.03 M NaCl/0.003 M sodium citrate, pH 7/0.1% SDS as described (16). RNA used for primer extension was isolated from PC-12 cells that had been treated with NGF (100 ng/ml) for 2 hr and 1 μg of poly(A)$^+$ RNA was used for primer extension (17). DNA fragments were cloned in pBluescript plasmids (Stratagene), sequentially deleted by using the Erase-a-Base system (Promega), and sequenced by Sanger's dideoxy method (18).

RESULTS

d5 cDNA Identifies a Multigene Family. We have described four NGF-induced genes from PC-12 cells and have shown that at least three of these genes were transiently induced at the transcriptional level (12). We chose to study further one of these genes, *d5*, which has several interesting regulatory characteristics: (*i*) d5 cDNA transcription was induced in the absence of new protein synthesis, (*ii*) a decrease in d5 transcription rate required continued protein synthesis, and (*iii*) *d5* was not induced by cAMP; furthermore, cAMP repressed the induction by NGF. To further study the regulatory region of *d5* and to define its product(s), we isolated additional cDNA clones from the cDNA library using the original d5 cDNA clone as a probe (12). The total length of d5 cDNA (about 5.7 kb) was represented in overlapping cDNA clones, although no one clone had a full-length cDNA. As shown in Fig. 1, the overall restriction patterns of the different d5 cDNA clones that we had isolated were almost identical with two exceptions: (*i*) a *Hin*dIII site was not present in the original d5 clone but was present in other clones that contained this region (d5-24, d5-26, d5-27, d5-28, and d5-30), and (*ii*) the clone d5-29 was missing an *Eco*RI site that was present in other clones that contained this region (d5-27, d5-30, d5-30, d5-31, and d5-32). This suggests that

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TCTAACCATCTCTCCATCCTTTGTTATTAGTTGTTACTAGAAGCCTAGTGTCATTTGGA  d5-27

.....................T^........ C.....G.....................  d5
                              ......C...G.................*....  d5-30

GGCTGGTTCTCTTGAGAGGCAAAGCCACCAAGCTGACACTGAAGGGAGGTAGGTACTCC  d5-27
                      ........................................  d5-24
......C...**.......*.........................................  d5
.....................................................****......  d5-30

TTAAGGGAAAATCTAAGCCCAGAGAGACAGCCGGTAACAGCTAGGCTGCCTCTCTGCTC  d5-27
.....................................................*........  d5-24
................AT.............A.......A..........TTTTTGGC  d5
................T.....................A.................  d5-30

GCCTTTCTGTTTCATAGACAAAGCTTGTGCTTATATTTACAGTGGCCTTTTTGTCCAAA  d5-27
.....................................................  d5-24
TT.....................................................G  d5
.......................                                d5-30

AAAAGGCCTCCTGGTTTAGCTGTGTGACTAAATGCTATTTGTCCTCTTCAGTGGACCTC  d5-27
....A^.....................................................  d5-24
...**.....T.....................G....C.................  d5

TCTCCTCTAGATTCCCTTCTTGTTTTCTCACCATCCCACGTGAGATCGCTTGTTAGTAA  d5-27
.....................................................*.....  d5-24
.ATTCTCAAGATTC.....................TT......*.............  d5

CTGTTACAGGTCTTCTTTACCACCGAGGGAAAGACAGAATCCTACTAGAGGCCTGAAGA  d5-27
.......................                                d5-24
.....................T.............................A.....  d5
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FIG. 2. Sequence comparison of four cDNA clones in the region of the first *Hin*dIII site from the 5' end of cDNA (indicated in Fig. 1). The 413-bp sequence of d5-27 is shown. A dot in any sequence indicates that the base of that particular cDNA is the same as that of d5-27. Differences in the DNA sequence of d5, d5-24 and d5-30 from that of d5-27 are indicated below each base that has been altered. An underlined letter indicates deletion of the base in that particular cDNA clone, while a circumflex indicates the insertion of the base after the underlined clone.

either *d5* defines a multigene family or different d5 mRNAs are generated from the same gene by differential splicing.

To help distinguish these two possibilities, we sequenced an overlapping region of four independently isolated cDNAs. There were significant sequence variations among the different cDNAs (Fig. 2). Most of them were single-base changes, but small deletions and insertions were also observed with one major substitution of sequences spanning almost 100 bases in the case of the original d5 cDNA. This pattern of heterogeneity suggested that the different cDNAs were products of different genes rather than products of alternative splicing events.

Genomic Southern analysis showed that a probe to the 5′ end of the cDNA (the original d5 cDNA) hybridized to multiple bands of the PC-12 DNA, but with varying intensities (Fig. 3). The heterogeneous hybridization pattern of *Eco*RI, *Hin*dIII, and *Bam*HI-digested PC-12 DNA suggested that these restriction sites must be quite varied near the 5′ end of the gene. On the other hand, only a few bands hybridized strongly with *Kpn* I and *Sst* I digestion, suggesting that *Kpn* I and *Sst* I sites may be quite conserved. We concluded from this result that the heterogeneity in the restriction pattern in the d5 cDNA clones is not because d5 mRNAs are produced by differential splicing but because d5 is a multigene family. The genomic Southern blot was also consistent with the isolation of many more positive phage clones during the screening of a rat genomic library that would be expected for a single-copy gene (see below). Sequences homologous to d5 are detected on Southern analysis of DNA isolated from rat, but not from DNA isolated from a number of other species including mouse, human, quail, *Xenopus*, and *Drosophila* (data not shown). This is consistent with the observation that most retroviruses are found in only a single species or very closely related species.

Sequence Homology Between the d5 Gene Family and Rasheed Rat Sarcoma Virus. To define the gene product of *d5*, we sequenced the original d5 cDNA and used the sequence information to search for homology between *d5* and any known DNA sequences, using the data bank of the National Biology Research Foundation. We observed a striking homology (over 65% identical nucleotides) between the 5′ end of the *d5* sequence (d5-23, d5-28, and d5) and a partial sequence of the Rasheed rat sarcoma virus (13). The homologous region includes the several important features of a retroviral LTR including a primer binding site, a poly(A) signal, and a poly(A) addition site (Fig. 4). The poly(A) addition sites and signals are present in the 5′ region of the cDNA, but they are presumably functional only in the 3′ LTR, where they are also present (Fig. 5). The region of homology ended at the region of the *gag-ras* fusion gene of Rasheed rat sarcoma virus, suggesting that the endogenous virus from which the Rasheed rat sarcoma virus is derived is related to d5. The LTRs of the d5 cDNA are also homologous to a number of retroviruses including both feline and murine leukemia viruses (which had *k*-tuple algorithm scores as high as 52), although these homologies are less striking than those to the Rasheed rat sarcoma virus (which had a *k*-tuple algorithm score of 60). Thus, we conclude that the genes that encode the cDNAs d5, d5-30, d5-24, and d5-27 appear to be members of a family of retrovirus elements. We have named this multielement family, which is defined on the basis of homology to *d5*, the NICER family (for NGF-inducible cAMP-extinguishable retrovirus-like); and we will refer to members of this family as NICER elements.

Isolation of a NICER Element and Characterization of Its LTRs. Many of the essential regulatory elements of a retro-

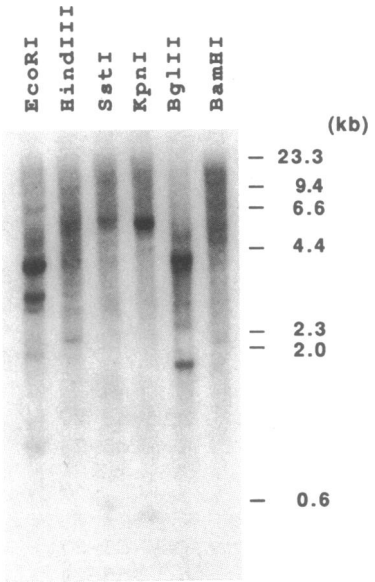

FIG. 3. Genomic Southern blot of PC-12 DNA. PC-12 DNA (20 μg) was digested with the restriction enzymes indicated in the figure, size-fractionated in a 1% agarose gel, transferred to nitrocellulose, and hybridized to labeled cDNA from the original d5 cDNA. Phage λ DNA digested with *Hin*dIII was used as a size marker.

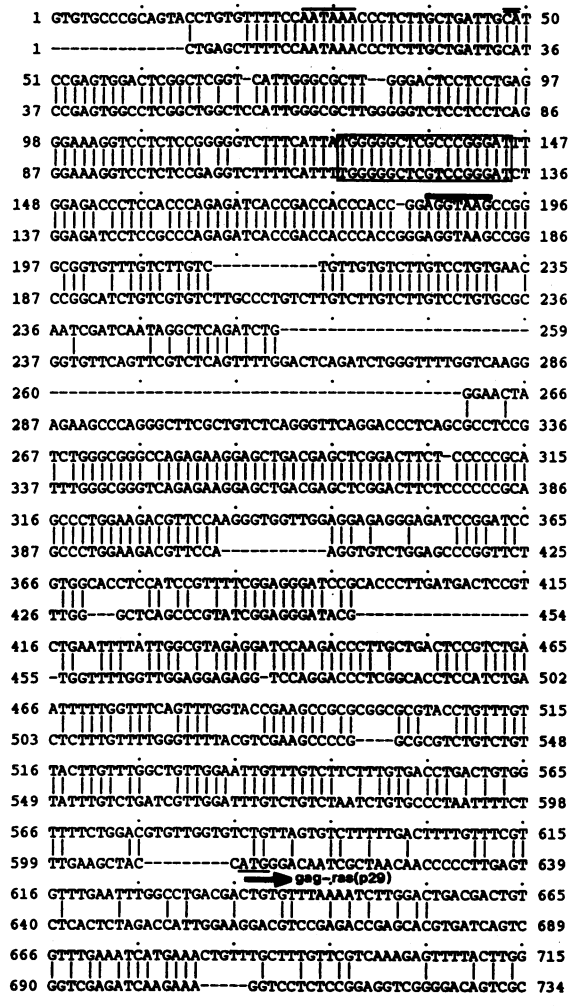

FIG. 4. Homology between the 5′-end sequence of Rasheed *ras* sarcoma virus (RaSV; lower sequence) and *d5* (upper sequence). The poly(A) signal (overlined), poly(A) addition site (double overlined), primer binding site (boxed), and 5′ splicing site (bold overlined) are indicated. Assignments of these regions are based on the sequence of RaSV (13). The initiation codon of the *gag-ras* fusion gene, which encodes p29 *ras*, is underlined.

FIG. 5. Sequence of the 5′ and 3′ LTR of the genomic clone λGL5-14. The sequence of the 5′ LTR is shown, and differences between the 5′ and 3′ LTRs are indicated. A star means deletion of the base in the sequence of the 3′ or 5′ LTR. The SRE, CAT, TATA, poly(A) addition signal, and poly(A) addition site are underlined and labeled. The transcription initiation site of *d5*, which is determined by primer extension (Fig. 6), is indicated and labeled as nucleotide 1, and upstream sequences are assigned negative numbers. The sequence that corresponds to the oligonucleotide used for primer extensions is underlined with dots. LTR sequences are capital letters, and the sequences of adjacent genomic (i.e., rat-derived) DNA are small letters. The potential duplication site of the genomic DNA (gaag), which is in small letters at both the 5′ boundary of the 5′ LTR and the 3′ boundary of the 3′ LTR, is also underlined. The repeat region (R) of the LTRs is overlined. The 5′ boundary of R is the transcription initiation site, while the 3′ boundary of R is taken as the poly(A) addition site. The poly(A) addition signal and site are presumably functional in the 3′ LTR. The U3 region includes the part of the LTR that is 5′ to R, while the U5 region is the part of the LTR that is 3′ to R. The region corresponding to the tRNA primer binding site, which is just 3′ to the U5 region of the 5′ LTR, is in italics.

virus are contained in the LTR, but the LTR is not expressed as a continuous unit in the mRNA. To obtain both the 5′ and the 3′ LTRs of a NICER element and to initiate the study of its regulatory elements, we isolated genomic clones by screening a rat genomic library with three different probes as described. Consistent with the expectation that NICER is a multigene family, we found that there were 258 ± 32(6)-fold more positives than would be expected if NICER elements were present as only a single copy per genome equivalent, and we found that hybridization occurred with a significant variation in intensity. The precise number of NICER elements per genome equivalent can't be obtained from this data; but, on the basis of the heterogeneity of the restriction maps, the sequence analysis of independently isolated d5 cDNA clones, Southern analysis, and genomic screening, we conclude that there are several hundred genes in the d5 family and that many of these genes are transcriptionally active.

We obtained 13 independent genomic clones of the NICER elements and a majority of these genomic clones shared strong homology by restriction analysis (data not shown). We chose to further characterize one of the clones, λGL5-14, because restriction analysis suggested that this genomic DNA contained a complete NICER element. The LTRs were separated by 5.6 kb, suggesting that they were present in a single NICER element. This conclusion was supported by Southern analysis with 5′- or 3′-specific probes (data not shown) and by DNA sequencing (Fig. 5).

To further define the 5′ and 3′ LTRs in λGL5-14, a 2-kb *Bam*HI fragment containing the 5′ LTR and another 1.5-kb *Bam*HI fragment containing the 3′ LTR were subcloned and sequenced. Comparison of the sequence of the two *Bam*HI fragments revealed the almost identical sequences in the stretch of 565 bp, with no homology beyond this 565-bp region (Fig. 5). There were nine differences between the 5′ LTR and the 3′ LTR, including six base changes, one insertion, and two deletions. Several lines of reasoning support the idea that these 565-bp regions are LTRs of a NICER element. First, there is a sequence duplication (GAAG) both at the 5′ junction of 5′ LTR and at the 3′ junction of 3′ LTR. Such a duplication (4–6 bp) is characteristic of a retroviral insertion event and is thought to arise from a duplication of host sequence during insertion. Second, these LTRs are found as direct repeats that are separated by 5.6 kb, which accounts for the observed size of the mRNA. Third, more than 98% of the bases in the 3′ LTR are identical to those in the 5′ LTR, suggesting that these sequences were derived from one another and have subsequently diverged.

We determined the transcription initiation site of the NICER element by primer extension (Fig. 6). The 20-mer used in the primer extension experiment is indicated in Fig. 5, where the site of transcription initiation is numbered as base 1. Several features typical of a eukaryotic promoter are found upstream of the start site. We found a TATA box and CCAAT box at −35 to −39 bp and −92 to −96 bp, respectively (Fig. 5). In addition, a sequence (CCATAAAAGG) at −286 to −296, which is similar to the serum-responsive element [SRE; consensus sequence of CC(A or T)$_6$GG; refs. 19–22]. A SRE is known to be involved in the induction of *fos* by NGF (22) and is present upstream of the *d2/NGF1A* gene, which is also induced by NGF (23). We conclude that the NICER family is a family of retrovirus elements whose transcription is induced by NGF.

DISCUSSION

Because the *d5* gene is a member of a family, the data on the transcription of this "gene" (12) must be reconsidered. The mRNAs in this family that are induced by NGF fall into a relatively narrow range of molecular weights (about 5.7 kb) by (Northern blot) analysis, suggesting that the "coding regions" of the members of the family are of similar size (12). It is clear

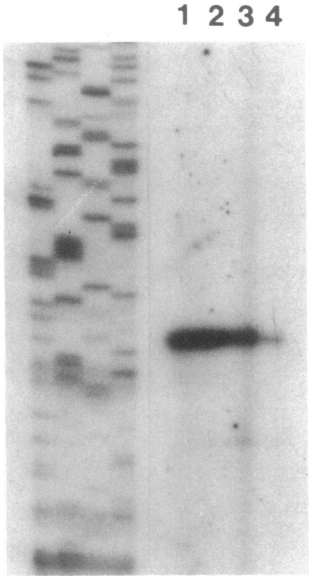

FIG. 6. Primer extension of d5 mRNA using a synthetic oligonucleotide (20-mer; 5′-GAGTCCACTCGGATGCAATC-3′) as a primer. This oligonucleotide was prepared based on the sequence data of the 5′-end sequence of the original d5 cDNA clone. The complementary base for the underlined G is missing in the underlined sequences in Fig. 5 because of the sequence variation between the d5 cDNA and the genomic clone, λGL5-14. Two independently prepared poly(A)$^+$ RNAs, samples A and B, were used with two different reverse transcriptases. Lanes: 1, poly(A)$^+$ (sample A) avian myeloblastosis virus (AMV) reverse transcriptase; 2, poly(A)$^+$ (sample B) AMV reverse transcriptase; 3, poly(A) (sample A) Moloney murine leukemia virus (Mo-MLV) reverse transcriptase; 4, poly(A)$^+$ (sample B) Mo-MLV reverse transcriptase. pBluescript SK$^+$ plasmid containing the 2-kb *Eco*RI fragment of the original d5 cDNA as an insert was used for double-stranded DNA sequencing to calculate the size of the primer-extended product (56 bases).

that multiple members of this family are transcriptionally active because we have isolated cDNAs that represent several different members of the family (Fig. 2 and unpublished sequence analysis from 10 different cDNA sequences). On the basis of the induction of the expression of several members of this family by NGF and the apparent repression of this transcriptional induction by cAMP, we can conclude that the regulatory elements in this family have been conserved (12). It is possible that many members of the family may have lost the response to NGF and/or cAMP, but that remains to be determined. The residual transcription that is seen in the presence of NGF and cAMP may be a result either of imperfect repression by cAMP or by the existence of some NGF-dependent transcription of elements that are not responsive to the inhibitory effects of cAMP. The regulation of the transcription of any one element can only be determined by the isolation of the regulatory regions of the DNA element so that the characteristics of that element can be measured without interference by RNAs produced by other members of the family. It is also possible that the NICER LTR may impose regulation on an adjacent gene. For example, the mouse *Slp* gene is apparently regulated by an LTR (7).

Although the NICER family has elements that are reminiscent of a retrovirus, the precise nature of the family has yet to be established; and the NICER element may be a retrotransposon, a competent retrovirus, a defective retrovirus, or a related DNA element. The induction of NICER expression by NGF and the repression of that induction by cAMP are unusual features of the family. We are not aware of any other retrovirus-like element with the same pattern of expression, but the VL30 element is somewhat similar in that it is positively regulated by epidermal growth factor and activators of protein kinase C (24–26).

The selective advantage of the regulatory pattern of the NICER elements is not clear; but, if the NICER element is capable of transposition, its expression must be tightly regulated or its insertion into host DNA will damage the host in most cases. The regulatory elements present in the NICER LTR could provide an important component of this regulation. If the NICER element is a transposable element, it is intriguing to consider the possibility that NGF, which is present in high concentration in the testes (27), may stimulate the expression of the NICER element in the germ line and thereby promote its passage to offspring.

We thank Robert Hawley for his initial involvement in isolating and characterizing cDNA clones and Paul Kaplan and Prescott Deininger for helpful advice and criticism. These studies were supported by a grant from the National Institutes of Health (CA22427 and 2S07RR05526-27).

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