

under different physiological states. Furthermore, a second group of neurons with α -MSH immunoreactivity has been discovered (25) and shown not to contain β -endorphin, a finding that would lead us to expect differential effects between the two peptides in other brain regions.

Nevertheless, our findings have implications for the understanding of endogenous mechanisms of pain modulation. Both opiate and nonopiate mechanisms of pain inhibition endogenous to the brain have been suggested (26). Our results demonstrate that these mechanisms can be produced by the action of two substances from the same neurons. Recent findings also indicate that analgesia arising from short-term stress is mediated by nonopiate mechanisms and that analgesia resulting from longer periods of stress is reversed by naloxone treatment (27). It is thus conceivable that α -MSH may participate in the former type of pain inhibition and β -endorphin in the latter. Finally, stimulation-produced analgesia is only partially reversed by naloxone (28). Since such electrical stimulation of pro-opiocortin-rich areas appears capable of releasing both α -MSH and β -endorphin (11), the residual analgesia may be due, in part, to the continued effects of α -MSH released from the same neurons.

J. MICHAEL WALKER
HUDA AKIL
STANLEY J. WATSON

Mental Health Research Institute,
University of Michigan, Ann Arbor 48109

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Transformation by Cloned Harvey Murine Sarcoma Virus DNA: Efficiency Increased by Long Terminal Repeat DNA

Abstract. *The coding sequences for the transforming (src) protein (p21) of Harvey murine sarcoma virus have been localized to a 1.3-kilobase pair segment near the 5' end of the viral genome. Ligation of the viral long terminal repeat DNA to the left end of the src region DNA markedly enhanced the low transforming efficiency of the src region DNA.*

We have been studying the structural and functional organization of the genome of Harvey murine sarcoma virus (Ha-MuSV), a C-type mammalian retrovirus that is highly oncogenic in vivo, induces focal transformation of fibroblasts in tissue culture, and is defective for replication. Infection by Ha-MuSV or other retroviruses results in the formation of infectious linear and circular viral DNA's. The unintegrated and integrated linear viral DNA's have direct long terminal repeat (LTR) sequences, which means that sequences at the left end of the viral DNA are identical to those at the right end (1). The LTR of the viral DNA is composed of sequences from both ends of the viral RNA genome; in each LTR, sequences that are derived from the 3' end of the viral RNA genome are located to the left of sequences that are derived from the 5' end of the viral RNA. The supercoiled viral DNA's, which represent circularly permuted forms of the unintegrated linear viral DNA, contain single or tandem copies of the LTR (1, 2). The function of the LTR's remains to be determined, but it has been speculated that they participate in the integration, transcription, and replication of the viral genome (1). In the studies reported here, we have employed transfection of Ha-MuSV DNA onto NIH 3T3 fibroblasts to show that the LTR enhances the efficiency of cellular transformation induced by the transforming (src) region of Ha-MuSV, al-

though the 0.65-kbp LTR of Ha-MuSV is not part of the src region.

The Ha-MuSV was originally isolated from a tumor that developed in a rat inoculated with Moloney murine leukemia virus (Mo-MuLV) (3); it is a recombinant between Mo-MuLV and rat cell nucleic acid sequences (4). The 5.5-kb RNA genome of Ha-MuSV is composed of an approximately 4.5-kb insert of rat sequences flanked on the 5' and 3' ends, respectively, by about 0.1 and 0.9 kb of Mo-MuLV sequences (5). The rat sequences of Ha-MuSV have a dual origin: (i) most are derived from endogenous retrovirus-like sequences that are expressed in some rat cells as 30S RNA (30S sequences) (6); (ii) those about 1 kb near the 5' end of Ha-MuSV are derived from sequences that are not found as 30S (7). Transformation by Ha-MuSV is associated with the production of a virus-encoded 21,000-dalton phosphoprotein (p21) which specifically binds guanosine diphosphate (GDP) and is the src protein of Ha-MuSV (8). Lower levels of an antigenically related p21 are present in normal cells from many species, as is also true of the src protein of Rous sarcoma virus (RSV) (9).

For a detailed examination of the Ha-MuSV genome, supercoiled Ha-MuSV DNA's have been molecularly cloned in *Escherichia coli* at the single viral Eco RI site, with λ and pBR322 vectors (10-12). Since the viral Eco RI site is located near the middle of the linear viral DNA,

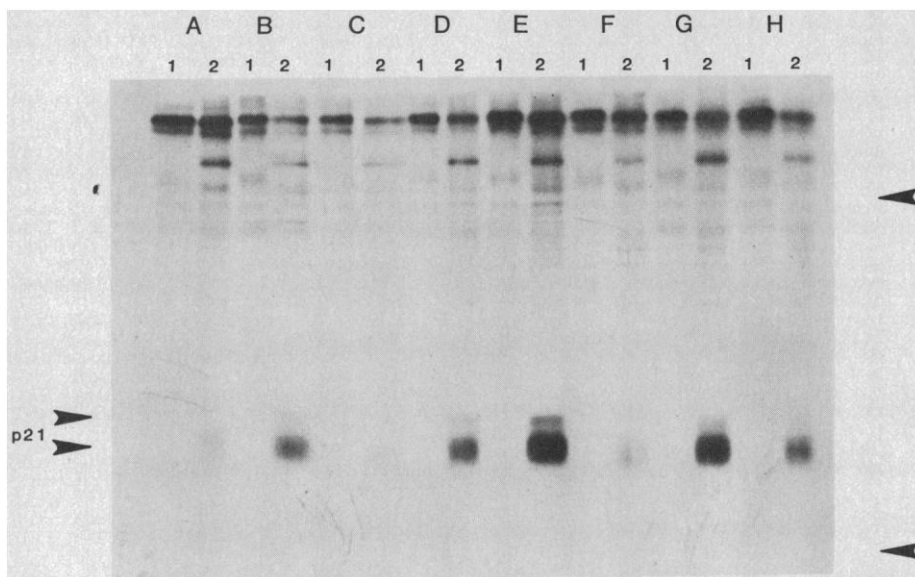


Fig. 1. Immunoprecipitation of Ha-MuSV p21 from extracts of NIH 3T3 cells transformed by Ha-MuSV. Cells were labeled with [35 S]methionine for 4 hours at 37°C, cell extracts were made, and immunoprecipitations were performed as described (8). Transformed NIH 3T3 cells used were (A) uninfected, (B and H) 2.1-kbp fragment, (C and F) 0.65-kbp fragment, (D and G) 0.65-kbp + 2.1-kbp fragments, and (E) transformed by Ha-MuSV virus. Serums used were (lane 1) normal rat serum and (lane 2) Ha-MuSV antiserum. The arrows indicate molecular weight markers: 68,000 (bovine serum albumin) and 14,000 (lysozyme).

the cloned viral DNA's are circularly permuted with respect to the linear viral DNA so that each molecule begins with the 3' half of the Ha-MuSV genome and continues through one or more copies of the LTR into the 5' half of the genome (see schematic diagram at bottom of Table 1). In spite of their permuted structure, the cloned viral DNA's efficiently transformed NIH 3T3 cells, even when the viral sequences were still covalently linked to the vector DNA. Defined subgenomic fragments and a series of insertion-deletion mutants of the cloned viral DNA have localized the transforming and the p21 coding regions of Ha-MuSV to a 2.3-kbp segment extending rightward from the 5' end of the nonpermuted linear viral DNA to the right-hand viral Pst I site shown in Table 1 (11, 12). Consequently, this segment contains one copy of the LTR and 1.7 kbp of rat sequences, including the entire 1 kbp of rat sequences other than 30S. This 5' location of the Ha-MuSV transforming region is different from the locations of Mo-MuSV and RSV transforming regions, which are at the 3' end of their genomes (13, 14).

In order to study the functional relationship between the LTR and the rat sequences in this 2.3-kbp segment, we first determined that interruption of the LTR greatly lowered the transforming efficiency of the viral DNA. Two cloned Ha-MuSV DNA's with one copy of the LTR were used for this experiment: a full-length 5.4-kbp Ha-MuSV DNA

cloned at the viral Eco RI site and a 3.8-kbp subgenomic Ha-MuSV fragment with viral Pst I sites at its termini (see schematic diagram at bottom of Table 1) (15). After Pst I digestion, the specific in-

Table 1. Influence of restriction enzyme digestion on infectivity of Ha-MuSV DNA. Restriction endonuclease digested viral DNA's (0.01 to 1 μ g per dish) along with calf thymus carrier DNA (25 μ g/ml) were transfected onto NIH 3T3 cells (11, 12, 21). Foci were counted 2 weeks later. The results are the average of two experiments. The infectivity is given as number of focus-forming units per microgram of viral DNA. The schematic diagram shows the physical map of the full-length, circularly permuted Ha-MuSV DNA cloned at the viral Eco RI site. The subgenomic clone, with viral Pst I sites as its termini, is represented by the heavy line. Abbreviations: E, Eco RI; Hc, Hinc II; K, Kpn I; P, Pst I; and X, Xba I.

Starting DNA	Enzyme	Infectivity
Full-length clone	Eco RI	4475
Full-length clone	Eco RI + Pst I	940
Subgenomic clone	Pst I	845
Subgenomic clone	Pst I + Hinc II	290
Subgenomic clone	Pst I + Xba I	15
Subgenomic clone	Pst I + Kpn I	8
pBR322	Pst I	0



fectivity of both clones was about one-fifth that of the intact full-length clone (Eco RI digestion), which implies that the 1.7 kbp of Ha-MuSV sequences (derived from 30S rat sequences) deleted in the subgenomic clone may influence the transforming efficiency of the viral DNA (Table 1). Digestion with Hinc II, which cleaves the subgenomic clone once at a site about 0.15 kbp to the left of the LTR, resulted in a two- to threefold loss in infectivity of the subgenomic clone. Digestion with Xba I, which cleaves only 0.5 kbp to the right of the Hinc II site but within the LTR, resulted in a more than 50-fold decrease in infectivity. Digestion with Kpn I, which cleaves within the LTR and 0.4 kbp to the right of this site, also gave a very low rate of transformation. This type of experiment could not establish whether the few residual foci that appeared after Xba I or Kpn I digestion were the result of incomplete digestion or of residual transforming activity of the resulting fragments. Nevertheless, these results demonstrate that cleavage within the LTR decreased the transforming efficiency of the subgenomic clone.

To determine whether sequences to the right of the Kpn I site located outside the LTR encode the viral p21 sequences, we used the full-length Ha-MuSV DNA insert to purify by gel electrophoresis a 2.1-kbp viral fragment that extended from this Kpn I site to the Eco RI site on the right (the 2.1-kbp fragment shown in Table 2). This fragment, which lacked the LTR, induced a rare transformant that contained p21. We therefore assessed the relative contribution of the LTR to the transforming efficiency of this 2.1-kbp fragment. The LTR, circularly permuted at the Kpn I site located within the LTR, was isolated by Kpn I digestion of a cloned Ha-MuSV DNA that contained tandem copies of the LTR (the 0.65-kbp fragment in Table 2). The 0.4-kbp Kpn I fragment that contained the sequences located between the Kpn I site in the LTR and the Kpn I site that forms the left end of the 2.1-kbp Kpn I-Eco RI fragment was also prepared. Ligation of the 0.65-kbp permuted LTR to the left end of the 2.1-kbp fragment resulted in a 20- to 30-fold enhancement in the transforming efficiency of the 2.1-kbp fragment ligated either to itself or to the 0.4-kbp fragment (Table 2). When individual foci were assayed for p21, those cells transformed with the 2.1-kbp fragment alone or with the LTR ligated to this fragment contained increased amounts of p21 (Fig. 1) which bound GDP; they also contained increased amounts of RNA from the 2.1-kbp frag-

ment but not from the 0.4-kbp Kpn I fragment or from the 3' half of the viral DNA (data not shown). As expected, cells derived from foci transformed by the permuted LTR alone did not contain increased p21 (or viral RNA) levels. These results confirmed the localization of the p21 coding region to the 2.1-kbp fragment.

Since the 2.1-kbp fragment and the 3.8-kbp subgenomic clone both contain the p21 coding sequences, we have inferred that the p21 coding region (whose minimum size should be about 0.6 kbp) is located within the overlapping sequences of the two segments, which are the 1.3 kbp between the Kpn I and Pst I sites shown by the heavy line in the diagram of Table 2. This region is derived exclusively from rat cell sequences and includes all the sequences other than 30S. The very low rate of transformation by the 2.1-kbp *src*-containing fragment is similar to those reported for analogous RSV and Mo-MuSV DNA fragments, where a low transforming efficiency has been demonstrated for viral DNA that lacks an intact LTR and other non-transforming viral sequences (14, 16, 17).

Our results show that ligation of the permuted Kpn I LTR to the left end of the 2.1-kbp fragment restored the transforming efficiency of the DNA to a level approximating that of the Hinc II-digested subgenomic clone. This enhancement is not simply the result of reestablishing the continuity of the sequences to the left of the *src* region, since the 0.4 kbp between the viral Kpn I sites have been deleted. These data demonstrate that the *src* region and the LTR are the principal determinants of the transforming activity of the Ha-MuSV DNA. The same may also be true for other retroviruses.

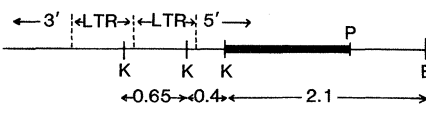
In Mo-MuSV, there is a high transforming rate (less than tenfold lower than that of intact full-length linear viral DNA) when subgenomic fragments contain a left-hand or right-hand LTR, the transforming region, and all the intervening nontransforming sequences in continuity (14, 16, 18). By contrast, for the cloned, circularly permuted, full-length Ha-MuSV DNA with one LTR, we previously found that when the LTR was located to the left of the *src* region the transforming efficiency was about 500-fold higher than when it was to the right of the *src* region (11, 12).

The mechanism by which the LTR increases the transforming efficiency of the p21 coding region remains to be elucidated. It is theoretically possible that the LTR facilitates integration of the viral DNA into host [or carrier (19)] DNA. We

consider this unlikely because the viral DNA in cells transformed by the circularly permuted Ha-MuSV DNA is integrated in the permuted orientation (as monomers or as dimers), rather than being integrated via the LTR, as probably occurs for unintegrated linear viral DNA with an LTR at each end (11, 12). We consider it most likely that the LTR has increased the efficiency of transformation by providing a promoter for viral RNA transcription. In support of this hypothesis we have previously shown that, following transformation by the cloned viral DNA, those transformants that contain viral DNA only in the circularly permuted monomeric form do not usually contain increased levels of RNA from those viral sequences located to the left of the LTR, although they invariably contain high levels of viral RNA from those sequences located to the right of the LTR (11, 12). The rare transformant that occurs after transfection with the 2.1-kbp fragment alone would presumably be the result of the inadvertent integration of this fragment next to a cellular promoter or the presence of a weak promoter in the *src* region. Regardless of the mechanism, our results imply that sequences in the LTR to the left of the Xba I site, which is located in that part of the LTR derived from the 3' end of the viral

Table 2. Enhanced transformation after ligation of the long terminal repeat to the p21 coding region. The fragments were prepared in two cycles of electrophoresis and electroelution of Kpn I + Eco RI-digested λ -Ha-MuSV DNA [clone 2 (10-12)]. The fragments were ligated (11, 12) at equimolar ratios and transfected (0.1 to 0.9 μ g of viral DNA per dish) before and after ligation. The results shown are the average of two experiments. The schematic diagram represents the right half of the Ha-MuSV DNA insert of clone 2, which contains two tandem copies of the LTR. Enzyme abbreviations are as in Table 1. The location of the three fragments prepared for ligation is also shown. The inferred p21 coding segment is represented by the heavy line between the Kpn I and the Pst I sites in the 2.1-kbp fragment.

DNA added	Focus-forming units per microgram of DNA	
	Unligated	Ligated
Full-length clone	4000	
2.1-kbp fragment	12	15
0.65-kbp fragment		5
0.65-kbp + 2.1-kbp fragments	6	410
0.4-kbp + 2.1-kbp fragments		25



RNA, are essential for enhanced infectivity (11, 12, 20).

Note added in proof: After submission of this report, Blair *et al.* reported (22) that the integrated LTR of Mo-MuSV enhanced the transforming efficiency of Mo-MuSV *src* DNA. In contrast to the results reported here, ligation of the integrated LTR to the *src* DNA was not required for this enhancement.

ESTHER H. CHANG

RONALD W. ELLIS

EDWARD M. SCOLNICK

DOUGLAS R. LOWY

Dermatology Branch and Tumor Virus Genetics Branch, National Cancer Institute, Bethesda, Maryland 20205

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