Harbor Symp. Quant. Biol. **44**, 643 (1979); H. Marquardt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4684 (1983).
18. R. C. Moyer, M. P. Moyer, M. H. Gerodetti, *J. Virol.* **26**, 272 (1978).
19. We thank D. Escobar, G. Estes, and Y. White for assistance and I. Cameron, W. Winters, and R. C. Moyer for valuable discussions and reviewing the manuscript. Supported in part by an institutional research grant from the American Cancer Society, grant RRO5654 from the National Institutes of Health, and the Department of Surgery, University of Texas Health Science Center at San Antonio.

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Duchenne Muscular Dystrophy Involving Translocation of the *dmd* Gene Next to Ribosomal RNA Genes

Abstract. *Duchenne muscular dystrophy (DMD) is a severe X-linked disorder leading to early death of affected males. Females with the disease are rare, but seven are known to be affected because of a chromosomal rearrangement involving a site at or near the* dmd *gene on the X chromosome. One of the seven has a translocation between the X and chromosome 21. The translocation-derived chromosomes from this patient have been isolated, and the translocation is shown to have split the block of genes encoding ribosomal RNA on the short arm of chromosome 21. Thus ribosomal RNA gene probes may be used to identify a junction fragment from the translocation site, allowing access to cloned segments of the X at or near the* dmd *gene and presenting a new approach to the study of this disease.*

Duchenne muscular dystrophy (DMD), the most common and severe of the muscular dystrophies, is an X-linked disorder normally affecting only males. However, the disease has been found in seven females, and in each case an X-autosome translocation is present in which the exchange point in the X is in band Xp21 near the middle of the short arm (*1–7*). As in most X-autosome translocations, the normal X chromosome in these patients is late-replicating and presumably inactive in all or most cells. None of the mothers have been shown to be carriers, and none of the parents carry the translocation. The concurrence of the repeated translocation with the Duchenne phenotype suggests that band Xp21 is the site of the *dmd* gene and that its activity is disrupted by the translocation. The nature of this disruption is unknown; in some cases it may involve direct interruption of the *dmd* gene while in others it may be due to action at a distance, perhaps involving conformational changes in chromatin at the site of the exchange. This, coupled with inactivation of the wild type (*dmd*$^+$) gene on the intact X chromosome, leads to the expression of the disease (*1–7*). This map location for the *dmd* locus is consistent with family studies that have shown linkage of the *dmd* locus to two restriction fragment length polymorphisms (RFLP's) flanking the Xp21 region (*8, 9*).

In the translocation case studied in our laboratory (*1*), the autosomal exchange point was found in the middle of the short arm of chromosome 21, a region known to carry multiple copies of the ribosomal DNA (rDNA) repeat unit coding for 18*S* and 28*S* ribosomal RNA (rRNA). Nucleolus organizer region (NOR) staining revealed that the translocation apparently split the block of ribosomal genes (*1*). We now report the isolation of both chromosomes derived from the translocation in somatic cell hybrids and show by molecular probing with human-specific rDNA sequences that rDNA is present on both translocation-derived chromosomes. This confirms that the rDNA block has been split by the translocation and suggests that rDNA probes might be used to detect molecular clones spanning the translocation site leading to isolation of the *dmd* gene, even though its product is not known.

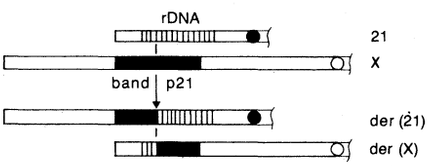

Fig. 1. Schematic of the short arms of chromosome 21, the X chromosome, and the two translocation products der(21) and der(X).

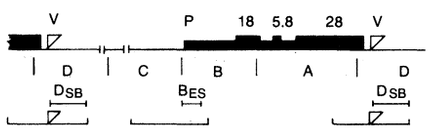

Fig. 2. Schematic of the rDNA repeat unit (*14*), approximately 43 kb in length. The transcribed portion is about 14 kb in length beginning at the promoter P and including the 18*S*, 5.8*S*, and 28*S* rRNA genes. Nontranscribed spacer separates the transcribed units. A variable region V contains one or more copies of a 900-bp internal repeat unit, generating length variability in this region (*15*). The main repeat unit is cut four times with Eco RI, giving four fragments: A (7.3 kb), B (5.7 kb), C (12 kb), and D (19 kb). Below the schematic are the subcloned regions D_{SB} and B_{ES} used as probes. The B_{ES} probe is an Eco RI–Sal I subclone from the B fragment (*16*), and the D_{SB} probe is a Sal I–Bam HI subclone from the D fragment (*15*); both are cloned into the appropriate site in pBR322. At the bottom of the figure are the Bam HI restriction fragments recognized by these probes. The Bam HI fragment recognized by D_{SB} is variable in length because it contains internal repeats.

The subject of our study is a 20-year-old female who was diagnosed at age 8 with Duchenne muscular dystrophy. Details of her phenotype and karyotype have been reported (*1*). In the schematic of her translocation (Fig. 1) we assumed that the ribosomal gene block was split by the translocation.

To test for the presence of rDNA on the translocation-derived chromosomes der(X) and der(21), we first separated them from human chromosomes 13, 14, 15, 21, and 22, each of which carries multiple copies of the rDNA repeat unit. This was accomplished by fusing the patient's cells to a mouse cell line and allowing the human chromosomes to be lost from the hybrid lines.

Hybrids were generated by Sendai virus–induced fusion of the patient's fibroblasts with mouse A9 cells deficient in hypoxanthine phosphoribosyltransferase (HPRT). Hybrids were selected in HAT medium (alpha medium containing 10 μg of hypoxanthine per milliliter, 10 μg of thymidine per milliliter, and 1 μ*M* methotrexate) containing 3 μ*M* ouabain, a dose sufficient to kill human cells but not A9 cells or hybrids (*10*). Colonies were picked from selective medium and grown continuously in HAT medium.

After being cultured for about 1 year in HAT medium, all hybrid lines retained the der(X) chromosome since the only active *hprt*$^+$ gene is on the long arm of this chromosome. Three independent hybrid clones were selected for further study. Hybrids A2 and F1 each contained only the der(X) translocation product. Hybrid C2 contained both der(X) and der(21) plus one or two other nonacrocentric human chromosomes. Subclones A2-4 and F1-3 were generated by plating A2 and F1 in HAT medium, and subclones A2-T4, F1-T5, and C2-T10 were generated by plating in medium containing 6-thioguanine to select for subclones that had lost the der(X) chromosome (*11*). Clones A2-4 and F1-3 contained the der(X); A2-T4 and F1-T5 had no human chromosomes; and C2-T10 contained only the der(21) and in some cells a human chromosome 5.

Further evidence for the presence of the der(X) chromosome in hybrid A2 and for the der(21) chromosome in hybrid C2-T10 was obtained from a study of X-linked markers. Hybrid C2-T10 was positive for human steroid sulfatase and for DNA sequences complementary to short arm probes DXS39 and DXS16 on the X chromosome; the hybrid A2 was positive for human G6PD and for the long arm probe DXS40 and centromere probe DXZ1 on the X chromosome (*12*).

To examine the der(X) and der(21) chromosomes for the presence of rDNA, human-specific rDNA probes were used. The rRNA genes are repeated 300 to 400 times in the human genome (*13*). The rDNA repeat unit (*14*) and the B_{ES} and D_{SB} probes derived from it are shown in Fig. 2. The D_{SB} fragment is from the nontranscribed spacer (*15*), while B_{ES} includes both transcribed and nontranscribed spacer sequences (*16*). Neither probe showed any significant cross-hybridization to mouse DNA under our hybridization conditions.

To test for the presence of human rDNA on the der(X) and der(21) chromosomes, DNA was prepared from each of the hybrid clones and subclones, restricted with Bam HI, run on agarose gels, blotted to nitrocellulose, and hybridized with ^{32}P-labeled rDNA probe. The D_{SB} probe revealed a band at 7.6 kilobases (kb) in hybrids A2 and F1 but not in mouse A9 (Fig. 3). The intensity of the band in A2 and F1 was less than that in human DNA at a 100-fold dilution. In human DNA there was a major band at 7.6 kb with other minor bands, showing that the Bam HI fragment revealed by D_{SB} contained the variable region (*15*) (Fig. 2).

The B_{ES} probe revealed the expected 6.3-kb band in DNA from the patient and her father (Fig. 4). The band was present in hybrids A2 and F1 and in subclones A2-4 and F1-3 but not in subclones A2-T4 and F1-T5 selected in 6-thioguanine. Since the subclones A2-T4 and F1-T5 have lost their der(X) chromosome, this result shows that the rDNA sequences present in the hybrids are carried on the der(X) chromosome and are not a result of a minor population of cells carrying one or more human acrocentric chromosomes. The intensity of the band from hybrid cells was less than that from 100-fold less human DNA but greater than that from 400-fold less human DNA. Assuming that there are 300 to 400 copies of the rDNA repeat unit in human diploid cells (*13, 14*) and correcting for the hybrids having about 50 percent more DNA per cell, we estimate that the der(X) chromosome carries three to five copies of the rDNA repeat unit.

In the hybrid C2-T10, which carries the der(21) but not the der(X), rDNA sequences were present (last lane of Fig. 4). The intensity of the 6.3-kb band was approximately equal to that from tenfold less human DNA; this is consistent with there being 30 to 40 copies per DNA equivalent or, correcting for the hyperdiploid nature of the hybrid cell, approximately 40 to 60 copies per der(21) chromosome.

These results show that the hybrids A2 and F1 are ideal sources of DNA from which to attempt cloning of a DNA segment spanning the translocation site. The der(X) chromosome carried in each of these hybrids contains three to five copies of the ribosomal gene repeat unit, flanked on one end by DNA from the distal end of chromosome 21 and on the other end by X-chromosome DNA at or near the *dmd* locus (Fig. 1). By Southern blot analysis (*17*) with rDNA probes and appropriate restriction digests, visualization of a junction fragment from each end of the cluster should be possible, such fragments being of different size and reduced intensity compared to the main band. Visualization of the X-junction fragment is a major first step in a rational approach to cloning this fragment and therefore in gaining access to the region of the X chromosome responsible for Duchenne muscular dystrophy.

In Fig. 4 a minor band is present in some lanes at 15 kb and is a candidate for a junction fragment. This minor band is present in hybrids A2 and F1 but not in C2-T10. It is also present in normal human DNA from both the patient and her father. From the relative intensity of the band it appears to be present in the hybrids A2 and F1 at about one copy per cell and in human DNA at about ten copies per cell. Since the patient's father does not have the translocation, this minor band cannot be the X-junction, but it could be the natural junction at the other end of the complex if this junction is the same in all ten rDNA-containing chromosomes. This interpretation is supported by the observation that the putative junction fragment is not present in DNA from the der(21) chromosome (hybrid C2-T10) but is present in several human DNA samples analyzed (data not presented).

Ongoing experiments to identify and clone the X-junction will provide access

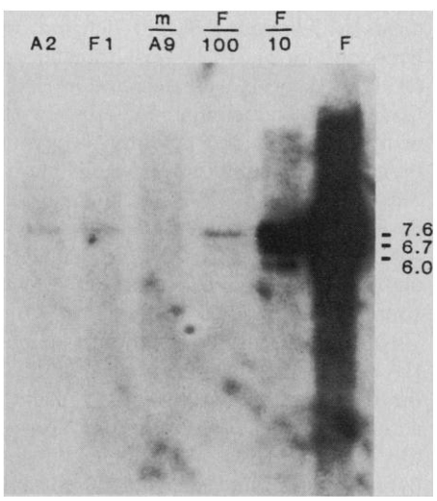

Fig. 3. Autoradiogram of Southern blot (*17*) with ^{32}P-labeled pD_{SB} as probe. High molecular weight DNA was prepared (*18*) from hybrid cultures A2 and F1, from mouse A9, and from lymphoblasts derived from the patient's father (F). In lanes A2, F1, m/A9, and F, 5 μg of Bam HI–digested DNA was loaded. In lanes F/10 and F/100, F DNA was diluted 1:10 and 1:100 before loading. Electrophoresis was performed in 0.8 percent agarose for 3.5 hours at 75 V. DNA was blotted to nitrocellulose (Schleicher and Schuell) and hybridized to whole plasmid pD_{SB} that had been labeled with [^{32}P]dCTP (deoxycytidine triphosphate) to more than 10^8 count/min by nick translation (*19*). The nitrocellulose was washed with 0.1× standard saline citrate and 0.1 percent sodium dodecyl sulfate for 1 hour at 65°C and exposed to Kodak X-omat AR film for 3 days at −70°C.

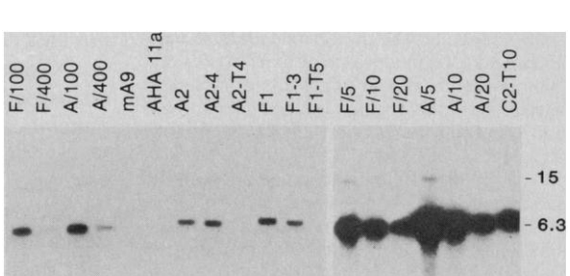

Fig. 4. Autoradiogram of Southern blot (*17*) with ^{32}P-labeled pB_{ES} as probe. High molecular weight DNA was prepared (*18*) from hybrid clones A2 and F1, subclones A2-4 and F1-3, thioguanine-selected subclones A2-T4, F1-T5, and C2-T10, mouse A9, AHA-11a [a hybrid containing a single human X chromosome and no other human chromosomes (*20*)], and lymphoblast cultures from the patient (A) and her father (F). In lanes 5 through 12 and 19, 5 μg of Bam HI–digested DNA was applied; in lanes marked F/*n* or A/*n* the same DNA (F or A) was diluted 1:*n* before loading. Electrophoresis was performed in 0.7 percent agarose for 3.5 hours at 75 V. Southern blot analysis was performed as described in the legend to Fig. 3.

to the region of the X chromosome that is somehow involved in the expression of Duchenne muscular dystrophy. DNA from this region will not only allow a rational approach to finding the *dmd* gene and its product but will also provide tightly linked probes that may be valuable for carrier detection and prenatal diagnosis in selected families.

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References and Notes

1. Ch. Verellen-Dumoulin *et al.*, *Hum. Genet.*, in press.
2. N. Canki, B. Dutrillaux, I. Tivadar, *Ann. Génét.* **22**, 35 (1979).
3. R. H. Lindenbaum, G. Clarke, C. Patel, M. Moncrieff, J. T. Hughes, *J. Med. Genet.* **16**, 389 (1979).
4. R. M. Greenstein *et al.*, *Cytogen. Cell Genet.* **27**, 268 (1980).
5. P. A. Jacobs, P. A. Hunt, M. Mayer, R. D. Bart, *Am. J. Hum. Genet.* **33**, 513 (1981).
6. M. Zatz, A. M. Vianna-Morgante, P. Campos, A. J. Diament, *J. Med. Genet.* **18**, 442 (1981).
7. B. S. Emanuel, E. H. Zackai, S. Tucker, *ibid.* **20**, 461 (1983).
8. J. M. Murray *et al.*, *Nature (London)* **300**, 69 (1982).
9. K. E. Davies *et al.*, *Nucleic Acids Res.* **11**, 2303 (1983).
10. L. H. Thompson and R. M. Baker, *Methods Cell Biol.* **6**, 209 (1973).
11. S. A. Farrell and R. G. Worton, *Somat. Cell Genet.* **3**, 539 (1977).
12. H. F. Willard, B. J. Schmeckpeper, K. D. Smith, S. J. Goss, *Cytogen. Cell Genet.* **37**, 609 (1984).
13. R. D. Schmickel and M. Knoller. *Pediatr. Res.* **11**, 929 (1977).
14. P. K. Wellauer and I. B. Dawid, *J. Mol. Biol.* **128**, 289 (1979).
15. J. M. Erickson, C. L. Rushford, D. J. Dorney, G. H. Wilson, R. D. Schmickel, *Gene* **16**, 1 (1981).
16. G. N. Wilson, L. Szura, C. Rushford, D. Jackson, J. Erickson, *Am. J. Hum. Genet.* **34**, 32 (1982).
17. E. Southern, *J. Mol. Biol.* **98**, 503 (1975).
18. H. F. Willard, K. D. Smith, J. Sutherland. *Nucleic Acids Res.* **11**, 2017 (1983).
19. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982).
20. B. P. Dorman, N. Shimizu, F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2363 (1978).
21. We thank K. Mellick and J. Sutherland for technical assistance; C. Verellen for assistance in obtaining tissue from the patient and her family; L. Siminovitch and L.-C. Tsui for critical comments on the manuscript; and F. H. Ruddle for providing the AHA-lla control hybrid cells. Supported by grants from the Muscular Dystrophy Association of Canada (R.G.W.), the Medical Research Council of Canada (R.G.W. and H.F.W.), and the National Institutes of Health (R.D.S.).
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Corticotropin-Releasing Factor Receptors in Rat Forebrain: Autoradiographic Identification

Abstract. *Corticotropin-releasing factor (CRF) receptors were identified in rat forebrain by autoradiography with an iodine-125–labeled analog of ovine CRF substituted with norleucine and tyrosine at amino acid residues 21 and 32, respectively. High-affinity receptors for CRF were found in discrete areas of rat forebrain, including laminae I and IV of the neocortex, the external layer of the median eminence, the lateral nucleus of the amygdala, and the striatum. These results are consistent with earlier findings on the immunohistochemical distribution of CRF and suggest that endogenous CRF has a physiological role in regulating activity of the central nervous system.*

Corticotropin-releasing factor (CRF), a 41–amino acid peptide originally isolated from ovine hypothalamus (*1, 2*), stimulates the release of proopiomelanocortin-derived peptides from the pituitary gland (*1, 3*). In addition to its endocrine activity in the pituitary gland (*1, 3*), CRF has extrahypophysiotropic effects. Intracerebroventricular administration of CRF elicits autonomic (*4–6*), electrophysiological (*7, 8*), and behavioral (*9–10*) effects, suggesting that it plays a crucial role in integrating the organism's response to stress. Radioimmunoassay (*11*) and immunocytochemical (*12*) studies have demonstrated that CRF-like immunoreactivity is distributed throughout the central nervous system (CNS), and pharmacological (*4–10, 13*) and immunohistochemical (*11, 12*) evidence suggests that CRF functions as a neurotransmitter or neuromodulator in the CNS. These actions of CRF in the brain are presumed to be initiated by binding to high-affinity receptors. Recently we identified and characterized high-affinity receptors for CRF in anterior and intermediate lobes of rat pituitary glands (*14*) and in the anterior lobe of human pituitary glands (*15*); however, specific CRF receptors in the brain had yet to be demonstrated. In the study reported here we used an iodine-125–labeled ovine CRF analog substituted with norleucine and tyrosine at amino acid residues 21 and 32, respectively, or [Nle^{21}, ^{125}I-Tyr^{32}]CRF, to identify receptor binding sites for CRF in sections of rat brain with light microscopic autoradiography.

We previously demonstrated that the kinetics, pharmacology, and distribution of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in the rat pituitary gland are correlated with the biological potency and sites of action of CRF, suggesting that the ligand labels the physiologically relevant CRF receptor (*14*). To define the characteristics of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in rat brain, we incubated serial, slide-mounted tissue sections with increasing concentrations of CRF-related and -unrelated peptides. Autoradiograms of these brain sections and iodinated standards were generated with tritium-sensitive Ultrofilm (LKB). An analysis of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in the striatum was performed by microdensitometry, and the film optical density was related to the molar concentration of radioactivity by use of the standard curve generated concomitantly with the autoradiograms (*16*). The binding of [Nle^{21}, ^{125}I-Tyr^{32}]CRF in rat striatum was saturable and on Scatchard analysis revealed a high-affinity component with an apparent dissociation constant of 6.2 ± 0.8 n*M* and maximum binding of 4.5 ± 1.5 fmole per milligram of protein (means ± standard errors; $n = 3$).

To characterize [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding pharmacologically, we examined the relative potencies of 1 μ*M* concentrations of CRF-related and -unrelated peptides in inhibiting specific binding to slide-mounted sections of rat forebrain. In these competition studies, 1 μ*M* rat CRF and ovine CRF inhibited 105.5 ± 6.3 and 80.8 ± 0.2 percent, respectively, of the specific [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in rat striatum. Two analogs of ovine CRF, CRF-(1–39) and CRF-(1–22), which are weak in stimulating proopiomelanocortin-derived peptide secretion from the pituitary (*1*), displaced 31.4 ± 0.9 and 29.4 ± 3.6 percent, respectively; the unrelated peptide, arginine vasopressin, did not significantly inhibit [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding at 1 μ*M*. These kinetic characteristics and pharmacological properties of the CRF binding site in rat striatum are comparable to those for the CRF receptor in rat anterior pituitary membrane homogenates (*14, 17*) and slide-mounted bovine pituitary sections (*14*), substantiating earlier suggestions (*13, 18*) that the structural requirements for CRF activity are shared by brain and pituitary receptors.

The autoradiographic mapping of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding sites in rat forebrain is shown in Fig. 1. Specific high-affinity binding sites for CRF were heterogeneously distributed, with the highest densities observed in laminae I and IV of the neocortex (Fig. 1, A and B) and in the external layer of the median