HRX Leukemic Fusion Proteins Form a Heterocomplex with the
Leukemia-associated Protein SET and Protein Phosphatase 2A*

(Received for publication, May 7, 1997, and in revised form, August 29, 1997)

Haskell T. Adler‡§, Ferez S. Nallaseth‡, Gernot Walter‡, and Douglas C. Tkachuk‡)

From the ‡Veterans Administration Puget Sound Health Care System, Seattle Division, Seattle, Washington 98108, the
‡Department of Pathology, University of California at San Diego, La Jolla, California 92037-0612, and the ¶Department of
Pathology, University of Washington School of Medicine, Seattle, Washington 98195-7470

One of the most common chromosomal abnormalities in acute leukemia is a reciprocal translocation involving the
HRX gene at chromosome locus 11q23, resulting in
HRX fusion proteins. Using the yeast two-hybrid system,
in vitro binding studies, and human cell culture coin-
munoprecipitation experiments, we show here that a region
of the HRX protein that is consistently retained in
HRX leukemic fusion proteins interacts directly with
SET, another protein implicated in leukemia. We have identified the binding sites on HRX for SET and show that
these sequences are clustered near the A′T hooks
that have been shown to bind DNA. We also show that
carboxyl-terminal SET sequences, possibly the acidic
tail of SET, bind to HRX. We have also found serine/threonine-specific protein phosphatase activity in anti-
HRX coimmunoprecipitates. Using the phosphatase in-
hibitor okadaic acid and Western blotting, the phosphatase was identified as protein phosphatase 2A (PP2A). Mutation of a single amino acid in one of the
SET binding sites of HRX resulted in lower amounts of
both coinmunoprecipitated SET protein and coinmu-
oprecipitated PP2A. These results suggest that the leu-
komogenic effects of HRX fusion proteins may be related
to interactions with SET and PP2A.

Disruption of the human homologue of the Drosophila
Trithorax protein, HRX, by chromosomal translocations to form
HRX fusion proteins is one of the most common genetic alter-
ations in human acute leukemia (1). These translocations occur in
approximately 10% of acute lymphoid leukemias, 5% of acute
myeloid leukemias, and 85% of secondary leukemias that occur
in patients as a complication of treatment with topoisomerase II inhibitors. Moreover, these translocations are present in half
of all the de novo leukemias in children less than 1 year of age
(2). Elucidating the role of HRX fusion proteins in the patho-
genesis of acute leukemia will increase our understanding of
the basic mechanisms controlling hematopoietic growth and
differentiation and will potentially identify molecules that will
serve as useful targets for therapeutic intervention in these
fatal diseases.

The HRX gene was cloned and found to encode a 3969-amino
acid protein that is also referred to as acute lymphoblastic
leukemia-1, MLL-1, or HTRX (1, 3–5). The gene spans approxi-
mately 90 kilobases and consists of 36 exons (6). HRX is a
nuclear protein that has at least two regions of strong
homology to the similarly sized Drosophila Trx protein; these
include a series of zinc fingers and a stretch of 210 amino acids
in the central and carboxyl-terminal regions of the protein,
respectively (see Fig. 1A). In Drosophila, Trx controls body
segment patterning as a positive transcriptional regulator of
the homeotic selector genes of the Antennapedia and bithorax
complexes (8). Studies with transgenic mice have shown that
the function of Hrx in mice has features in common with that of
trithorax in Drosophila. Yu et al. (9) have shown that Hrx is
required for proper segment identity and positively regulates
Hox gene expression in Hrx heterozygous and homozygous
deficient mice (9). It is generally thought that the Drosophila
Trx protein is part of a cellular memory system that is involved
in the stable inheritance of gene activity.

Some 25 different human leukemic HRX fusion proteins
resulting from reciprocal translocations between the HRX gene
at chromosome 11q23 and partner genes at other loci are pre-
dicted to exist from cytogenetic studies (10, 11). To date, at
least 13 of the partner genes have been cloned and character-
ized (12, 13, 64, 65). Cytogenic and Northern blot analyses on
patient specimens and leukemia cell lines consistently show
retention of the derivative 11 fusion product, suggesting that
this product is the critical leukemogenic factor (14). Recent
support for this assertion has come from transgenic mice,
where expression of a derivative 11 fusion product resulted in
leukemia (15). All of the derivative 11 fusion products, herea-
fter simply called HRX fusion proteins, are essentially com-
posed of common amino-terminal HRX sequences fused to a
variety of carboxyl-terminal residues donated from one of 25
partner proteins. The fusion partners are structurally and
functionally unrelated except for stretches of acidic residues
that are typical of transactivation domains. In at least one case,
an HRX fusion partner, the ENL protein has been shown to
activate transcription from synthetic reporter genes in both
human lymphoid and myeloid cells as well as in yeast (16).
Additionally, one HRX fusion partner, CBP, has a defined
enzymatic activity (the ability to acetylate histones), and this
activity is predicted to be present in the HRX-CBP fusion
proteins (64, 65). The HRX fusion partners likely play an im-
portant role in leukemogenesis, as neither simple truncated
versions of HRX nor HRX fusion proteins with a partner fused
out of frame have been found in leukemias (13). Studies that
support this idea have recently been done with HRX-ENL.
HRX-ENL is able to immortalize hematopoietic stem cells in
vitro. However, neither wild-type ENL nor a deletion mutant of
HRX-ENL lacking ENL had in vitro transforming ability (66).

Two other variant forms of HRX have been described that
are not associated with chromosomal translocations. Cases of

* This work was supported by Department of Veterans Affairs Re-
gional Administration Grant 0901 and an American Cancer Society
Institutional Grant (HRX/Interact) administered by the University of
Washington. The costs of publication of this article were defrayed in part
by the payment of page charges. This article must therefore be
hereby marked “advertisement” in accordance with 18 U.S.C. Section
1734 solely to indicate this fact.

§ To whom correspondence should be addressed: VA Puget Sound
Health Care System, Seattle Division, 1660 S. Columbian Way,
Research Mail Stop 151, Seattle, WA 98108. Tel.: 206-762-1010 (ext.
3072); Fax: 206-764-2598; E-mail: hadler@u.washington.edu.

This paper is available on line at http://www.jbc.org
HRX Associates with the SET Protein and PP2A

acute leukemia have been described where HRX has undergone partial duplication, resulting in leukemic HRX “self-fusion” proteins. These self-fusion proteins have a tandem duplication of amino-terminal sequences encoded by exons 2–6 or 2–8 that result in longer than wild-type proteins (17). Many of the duplicated amino-terminal sequences are the same as those found in HRX fusion proteins. Also, deletions of HRX exon 8 have been found in some T-cell acute lymphoid leukemias (18).

To date, an actual transforming capability for the self-fused or exon 8-deleted HRX proteins has not been demonstrated.

The HRX fusion proteins contain near their amino termini three closely spaced A'T hooks composed of conserved basic amino acids (see Fig. 1B). The A'T hooks were originally described as a DNA binding motif in the high mobility group protein, HMG-I(Y), a nonhistone chromatin-associated protein that preferentially binds to the minor groove of A'T-rich DNA (19). A'T hooks are unusual in that they recognize DNA structure rather than nucleotide sequence. Reeves and Wolff (20) have shown that HMG-I(Y) can preferentially bind certain types of A'T-rich DNA located on the surface of mononucleosomes. It has been found that nucleosomes positioned on critical regulatory sequences can repress transcription by making regions inaccessible to trans-acting factors (21). HMG-I(Y) itself has been termed an “architectural transcription factor” because not only can HMG-I(Y) directly bind other transcription factors, but also A'T hook binding to nucleosomal DNA appears to bend or distort promoter/enhancer DNA sequences, possibly making these sequences accessible and thereby overcoming the repressive effects of nucleosomes. Thus, HMG-I(Y) facilitates the binding of transcription factors to DNA and promotes the formation of efficient transcriptional initiation complexes (20, 22, 23). HRX A'T hooks also bind DNA. To date, HRX has been shown to bind cruciform DNA and scaffold attachment region (SAR) DNA (24, 25). This capacity for DNA binding suggests that both HRX and HRX fusion proteins function in the cell in part through direct protein-DNA interactions, possibly in a manner similar to HMG-I(Y) proteins.

At present little is known about the actual function of the HRX gene in hematopoietic development. Fidanza et al. (26) have shown that homozygous Hrx-deleted embryonic stem cells are blocked in hematopoietic differentiation in vitro. Neither a cognate DNA binding sequence nor a definitive targeted transcriptional unit has been described yet for HRX or HRX fusion proteins. The cognate DNA binding sequence nor a definitive targeted transcriptional unit has been described yet for HRX or HRX fusion proteins.

An attractive approach to understanding HRX gene function is to identify proteins that interact directly with HRX in vivo. Leshkowitz et al. (28) have found that a region common to the HRX fusion proteins (amino acids 1–1096) is capable of binding the UnR protein, a protein of unknown function that has a high affinity for single-stranded DNA or RNA (29). Data extrapolated from yeast have suggested that the HRX fusion partners ENL and AF-9 may interact with the human SNF5 component of the human SWI/SNF complex, a chromatin remodeling system (30). In this current study, we show that amino-terminal HRX residues associate with the SET protein and coimmunoprecipitate protein phosphatase 2A (PP2A) activity. Our findings suggest that HRX fusion proteins might function in conjunction with SET and PP2A to deregulate cellular growth and differentiation controls resulting in leukemia.

EXPERIMENTAL PROCEDURES

Cell Lines—Human 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (31).

Construction of Expression Vectors—Portions of HRX were cloned into the expression vector pCS2+MT, which allows for in-frame fusions with six copies of the myc epitope tag under the control of a simian cytomegalovirus promoter (32, 33). pCS884 was made by first cloning the Smal to Scal fragment of HRX (amino acids 110–405) into the Smal site of pBSK+. Then an XhoI to HincII fragment was cloned into the XhoI and Scal sites of pCS2+MT. pCS884 and pCS888 are chimeric inserts using pCS884 and pCS882 as starting materials. pCS1385EI and pCS1385Bpu both begin at the NorI site of HRX (amino acid 79) and continue to the BspEI (amino acid 329) and Bpu1102 (amino acid 370) sites, respectively. pCS1385 begins at the NorI site of HRX (amino acid 79) and continues to the XhoI site (amino acid 741). pCS8AR contains the entire HRX-ENL coding sequence beginning at the AvaII site at amino acid 27, blunted with Klenow fragment, and cloned into the StuI and EcoRI sites of pCS2+MT by a multi-step cloning procedure. pCSARXB is a derivative of pCSAR that expresses the same protein and contains a deletion of an XhoI site from amino acids 5–437 of the pCS2+MT vector. All other individual fragments were made using a polymerase chain reaction-based method and correspond to the amino acids shown in Fig. 2.

The pCSHMGI-1 vector was made with the NorI to XhoI fragment of pBSHMGI-1 (clone 7C) cloned into the Smal and Scal sites of pCS2+MT (34). The pCSHMGI-1 vector was made with the HindIII (filled-in) to XhoI fragment of HMG-I(Y) provided in pBBSK+ by K. Chada and cloned into the StuI and XhoI sites of pCS2+MT (35). pCSSETB3 was made with the EcoRI to NorI (filled-in) fragment of pBSK+127 cloned into the EcoRI and StuI sites of pCS2+MT. This clone contains a full-length myc-SET fusion protein with SET amino acids 1–278. Portions of SET were also cloned into the expression vector pSET-HT10 (mouse gene) that has been modified for in-frame fusions with the eight-amino-acid IBI FLAG™ marker peptide (Eastman Kodak Co.) (the kind gift of B. Schubach). The construct pSGSET was made with the BamHI to EcoRI fragment of pBSK+127 cloned into the BamHI and EcoRI sites of pSG5+FLAG. The construct pSGNSET was made with the BamHI to MscI fragment of pBSK+127 cloned into the BamHI and Smal sites of pSG5+FLAG. pSGSET and pSGNSET express FLAG-tagged full-length SET protein and an N-terminal 213-amino acid SET protein, respectively.

Construction of HRX Mutants—Mutations were introduced into a human HRX cDNA by incorporation of a phosphorylated oligo during polymerase chain reaction amplification as described by Scott Michael (36), with the substitution of 6 mM magnesium chloride for 10 mM magnesium acetate.

Yeast Two-hybrid Library Screening and Protein-Protein Interaction Assay—The yeast two-hybrid library screening was performed as described by Wu et al. (37). Briefly, the bait vector pBTM884 contains an in-frame protein fusion between LexA and HRX; the Smal to SapI 885-base pair fragment of HRX was cloned into the Smal site of pBTM116 (38). The pBTM116 vector contains the yeast TRP1 gene. The bait plasmid was used to transform the yeast strain L40 (MATa his d200 trp1-90 leu2-3112 ade2 lys2::[lexAop]-HIS3 URA3::[lexAop]-8-loxZ GAL4 gal80). Subsequently, the strain was transformed with a B cell cDNA library. The B cell cDNAs are expressed as fusion proteins with a functional GAL4 activation domain (amino acids 768–881) in the plasmid pSRE107 containing the yeast LEU2 gene. Yeast transformants containing interacting proteins were identified by their ability to grow on plates lacking tryptophan, leucine, and histidine.

In vitro Transcription-Translation—GST-HRX fusion proteins were produced in Escherichia coli XL-2 (Stratagene) using the pGEX884 and pGEX562 plasmids. pGEX884 was made as described above, and pGEX562 was made using the polymerase chain reaction amplification to produce a BamHI/NorI fragment coding for HRX amino acids 161–439, which was subsequently cloned into the respective sites of pGEX-4T-1 plasmid (Pharmacia Biotech Inc.). In vitro translated 35S-labeled SET protein was made using the TNT-coupled reticulocyte lysate system according to the protocol of the manufacturer (Promega) with 35S express label (NEK Life Science Products). The plasmid pBSK+127 that contains a cDNA for a β isofrom of SET was used. GST-HRX fusion proteins were immobilized on glutathione agarose beads (Sigma) and

1 The abbreviations used are: SAR, scaffold attachment region; PP2A, protein phosphatase 2A; colP, co-immunoprecipitation.
added to in vitro translated 35S-labeled SET protein in binding buffer (20 mM HEPES, pH 7.5, 10% glycerol, 12.5 mM MgCl₂, 0.1 mM EDTA, 50 mM NaCl). The agarose beads were washed three times with binding buffer. Proteins were resolved by polyacrylamide gel electrophoresis on 10% gels. The gels were treated with Amplify (Amersham Life Science, Inc.) and dried using a Bio-Rad model 583 gel dryer. Fluorography was performed with Kodak XAR-2 film at -70 °C.

**Coimmunoprecipitation and Western Blotting—**Human 293T cells were transiently transfected with HRX constructs using the calcium phosphate transfection method, grown for 2 days, and lysed in high salt lysis buffer (500 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 4 μg/ml leupeptin, 4 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin). After sonication and centrifugation, the lysates were immunoprecipitated with anti-myc mouse monoclonal 9E10 (gift from Jim Roberts, Fred Hutchinson Cancer Research Center, Seattle, WA) followed by protein A Sepharose CL-4B (Pharmacia). To allow binding of these proteins, we used a LexA-based yeast two-hybrid screen generously provided by Terry Copeland using 5% nonfat dry milk and 0.2% Tween-20 in phosphate-buffered saline. Western blotting of cell lysates was performed with the anti-myc mouse monoclonal 9E10 described above or with anti-HRX to set proteins. To detect primary antibodies, horseradish peroxidase-conjugated goat antibody to either rabbit immuno-globulin (IgG) or mouse IgG (Sigma) was used at a dilution of 1:5,000. Bands were visualized with enhanced chemiluminescence (ECL) reagents (Bio-Rad). Western blotting of co-immunoprecipitation (coIP) for PP2A was done as described previously by Kremmer et al. (39) using the 6G3 monoclonal antibody.

**Phosphatase Assays—**Cells were transfected with various constructs, lysed, and immunoprecipitated as described above. Phosphatase assays were performed as described by Li et al. (40) with minor modifications. Briefly, IPs were assayed in 50 mM Tris-HCl, pH 7.0, 10% glycerol, 2 mg/ml bovine serum albumin, 2 mM MgCl₂, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 14 mM β-mercaptoethanol with 20 μg of 32P-labeled histone H1 as substrate in a final volume of 50 μl. Reagents were added on ice, and reactions began by incubation at 30 °C; after 15 min, the reaction was terminated with 100 μl of 20% trichloroacetic acid. The mixture was centrifuged for 6 min, and 120 μl of the supernatant was counted by Cerenkov counting. The substrate, histone H1 (Boehringer Mannheim), was prepared as described by Li et al. (40), with the substitutions of 5 units/ml rat brain protein kinase C (Calbiochem) and 20% trichloroacetic acid as suggested by the authors. The final pellet was resuspended in a volume of 1 ml of 50 mM Tris-HCl, pH 7.0, 10% glycerol, 1 mM benzamidine.

**Phosphatase Inhibitor Assays—**The phosphatase inhibitor oedaic acid (sodium salt; Sigma) was dissolved in water, serially diluted, and stored at -20 °C. The inhibitor was added by dilution of the phosphatase reactions described above. PP2A, the generous gift of Marc Mumby was used as the standard in the assays.

**RESULTS**

**Yeast Two-hybrid Screen Reveals That HRX Binds SET—**To identify proteins that interact with HRX and therefore may participate in HRX fusion protein-mediated leukemogenesis, we chose to perform a yeast two-hybrid screen with a fragment of HRX-ENL that contains three A'T hooks. This fragment of HRX was selected because of its similarity to the small architectural transcription factor HMG-I(Y), a protein of only 100 amino acids that contains three zinc fingers and because HMG-I(Y) has been shown to have protein-protein binding sites near its A'T hooks. We therefore used HRX884 (Figs. 1A and 2), a polypeptide of 295 amino acids including three A'T hooks, to identify proteins that bind to the HRX protein. To identify these proteins, we used a LexA-based yeast two-hybrid screen (41, 42). Our " bait" was a fusion between the LexA DNA binding domain and amino acids 110–405 of HRX-ENL (pBTM884). The bait protein was used to screen an Epstein-Barr virus-transformed human B-lymphocyte cDNA library expressed as fusion proteins joined to an activation domain of GAL4 (amino acids 768–881). These two hybrids were expressed in *Saccharomyces cerevisiae* strain L40, which is able to grow in the absence of histidine if the two hybrid proteins interact, due to restoration of transcriptional activation of the HIS3 gene, which contains four upstream tandem LexA binding sites. Of approximately 450,000 TRP LEU transformants, 15 were also HIS. A secondary screen showed that all 15 also stained positive for expression of the *E. coli* β-galactosidase gene (data not shown). The L40 strain contains both reporter genes driven from different promoters each containing LexA binding sites. The 15 presumptive positives were also tested by growing yeast colonies in minimal medium containing tetracyphon, resulting in the loss of the DNA binding domain vector (pBTM884) from the reporter strain. Mating tests were then performed with strain AMR70 containing either pBTM884 or a LexA human lamin fusion protein (the human lamin protein displays a high degree of nonspecific binding). The diploid progeny were assayed for β-galactosidase activity. As a final test, library cDNAs in the pSE1107 plasmid were recovered from the yeast by transformation into *E. coli*. These plasmids were then reintroduced into the L40 yeast strain containing the bait vector pBTM884 to affirm activation of the two reporter genes. Of the 15 candidates, 13 passed these subsequent tests. 10 of the 13 were found to code for SET proteins (43). Both isoforms of SET (previously termed TAF-1α and TAF-1β) were recovered in our screen, four α and six β (44). These two proteins have different amino termini, 37 amino acids (α) and 24 amino acids (β) but are otherwise identical throughout the other 253 amino acids of their sequences (43). Interestingly, the SET protein is a member of a chimeric fusion protein, SET-CAN, that arises as a result of chromosome translocations in acute undifferentiated leukemia (45). A nearly full-length SET protein is present in the SET-CAN fusion with only the last
seven amino acids of SET lost upon translocation. SET has homology to the nucleosome assembly protein, NAP1, and has been shown to be an inhibitor of protein phosphatase 2A (46).

**HRX Binds SET in Vitro and in Vivo**—We confirmed the yeast two-hybrid results by in vitro binding studies using standard gel retention assays with purified bacterial GST-HRX884 fusion proteins and in vitro translated 35S-labeled SET protein. GST-HRX884 and a truncated version, GST-HRX562, (see Fig. 2 for constructs) were tested for their ability to interact with SET. Fig. 3A shows retention of the 39-kDa SET protein by GST-HRX884 in lanes 3 and 4, whereas the negative control GST and GST-HRX562 fails to bind SET in lanes 1 and 2 and lanes 5 and 6, respectively. These experiments show that the SET protein specifically interacts with GST-HRX884 but not GST-HRX562 and binds residues flanking the central A-T hook triplet in HRX884.

To investigate whether HRX associates with SET in vivo and to delimit the region of HRX that interacts with SET, a coIP procedure was developed in which portions of HRX tagged with the myc epitope (e.g. myc-HRX884) were expressed in human kidney 293T cells and immunoprecipitated with anti-myc epitope antibodies. Cells that expressed the myc-HRX constructs pCS1385 (HRX amino acids 79–741), pCSC884, and pCS884 coimmunoprecipitated endogenous SET protein strongly (Fig. 3B, lanes 5, 9, and 10), whereas the other myc-HRX constructs pCSAFAR, pCS1385Bpu, pCS562, and pCSN884 bound little or no SET protein (Fig. 3B, lanes 1, 2, 3, 4, and 8). These results suggest that an element resides between amino acids 370 and 404 that is necessary for strong SET binding to HRX constructs. These experiments confirmed our yeast two-hybrid and in vitro binding studies and suggest that the amino-terminal HRX sequences retained in the various leukemic HRX fusion proteins contain a region with dual DNA binding and protein-protein interaction functions. We have also found that the nearly full-length HRX-ENL protein (lacking only the 26 N-terminal amino acids and containing amino acids 27–1999) binds SET in coIP experiments (Fig. 4, lane 8).

The fact that a small amount of SET is seen in the HRX562 coIPs (Fig. 3B, lane 4) whereas no SET is seen in the GST-HRX562 in vitro binding reactions (Fig. 3A, lanes 5 and 6) may simply reflect the larger pool of endogenous SET available for binding in the coIP procedure. It is intriguing that HRX562 (Fig. 3B, lane 4) binds more SET than HRXN884 (Fig. 3B, lane 8), suggesting that an element may exist between amino acids 110 and 161 that prevents maximal SET binding to HRX.

Deletion and Mutagenesis Analyses Suggest That Two Lysine-rich Regions of HRX Interact with the Acidic Carboxy-terminal Tail of SET—We found two primary SET binding sites on HRX. These sites were mapped through coIP experiments with deletion constructs to two lysine-rich regions corresponding to amino acids 209–213 (KIKKK) and amino acids 382–389 (GAQQKKEK). The first SET binding region (amino acids 209–213) was delimited by the two deletion constructs, pCSGFCR and pCSHFAR, and the second SET binding region (amino acids 382–389) was delimited by the two deletion constructs, pCSGFCR and pCSGFBR (Fig. 2). These data indicate that two regions of HRX flanking the two carboxy-terminal A-T hooks are both necessary for maximum SET binding. Interestingly, the other A-T-hook family members, HMG-I(Y) and HMG-I-C, do not bind SET in our assays (Fig. 3B, lanes 6 and 7), showing that this is not a general feature of proteins that contain a triplet of closely spaced A-T hooks.

To determine the region of SET responsible for binding HRX, we made constructs that express a tagged full-length SET β isoform (SGSET) that runs at a higher molecular weight than endogenous SET protein using polyacrylamide gel electrophoresis and constructs that express a tagged carboxy-terminal deleted SET (SNGSET) that contains amino acids 1–213 of a SET β isoform and has a lower molecular weight than endogenous SET protein. These constructs were cotransfected with myc-HRX884 constructs, and proteins bound to HRX884 were coimmunoprecipitated using anti-myc antibodies. We found that SGSET and endogenous SET were both coimmunoprecipitated with HRX884 (Fig. 4, lane 3), and if SGSET was expressed at a high level, it completely replaced the endogenous SET found coimmunoprecipitating with HRX884 (data not shown). We also cotransfected SGNSET and found that al-
Fig. 3A. HRX residues adjacent to the A/T hook DNA binding motifs bind the SET protein in vitro. Fluorogram of a polyacrylamide gel of bound 

tagged myc-HRX constructs (Fig. 5). Reducing activity was found coimmunoprecipitated with various myc-tagged SET proteins listed here. The constructs transfected are as follows (see Fig. 2): lane 1, pCSEFAR; lane 2, pCS1385E1; lane 3, pCS1385Bpu; lane 4, pCS662; lane 5, pCS1385; lane 6, pCSHMGI-Y1; lane 7, pCSHMGI-C; lane 8, pCSN884; lane 9, pCSC884; lane 10, pCS884. Lanes 5 and 7 are shown at a longer exposure to the right.

Fig. 4. Mutated HRX constructs bind less SET, and mutated SET constructs do not bind HRX. The myc panel is a Western blot of cell lysates using an anti-myc antibody to myc-tagged HRX deletion constructs. The SET panel is a Western blot, using an anti-SET antibody, of coIPs of the myc-tagged proteins expressed from the constructs listed below. Lanes 1, 2, 10, 11, and 12 are cell lysates. Lanes 3–9 show coimmunoprecipitated endogenous SET and tagged SET proteins with the myc-tagged proteins expressed from the constructs listed here. The constructs transfected are as follows (see Fig. 2): lane 1, pCS884 and pSGSET; lane 2, pCS884 and pSGSET; lane 3, colIP pCS884 and pSGSET; lane 4, colIP pCS884 and pSGSET; lane 5, colIP pCS2MT; lane 6, colIP pCSEFAR; lane 7, colIP pCSMUT-1; lane 8, colIP pCSARKB; lane 9, colIP pCS2MT; lane 10, pCS2MT; lane 11, pCSEFAR; lane 12, pCSMUT-1. Lanes 1–4, 5–7, 8 and 9, and 10–12 are four separate experiments. Lanes 10, 11, and 12 are the lysates that correspond to the colIPs in lanes 5, 6, and 7.

though it was expressed at a high level (Fig. 4, lane 2), it was not coimmunoprecipitated with HRX884 (Fig. 4, lane 4). These results suggest that the region of SET that is binding HRX is not wholly present in the amino-terminal 213 amino acids of SET. Instead, the HRX binding region is likely present in the remaining 64 amino acids of SET that contain an acidic tail composed primarily of aspartic acid and glutamic acid residues.

The two SET binding regions of HRX contain a number of positively charged lysine residues, suggesting to us that the negatively charged carboxyl-terminal tail of SET may be interacting with the positively charged lysine residues of HRX. To show that the SET-HRX interaction is not the result of a nonspecific interaction of oppositely charged residues, we created the clone pCSMUT-1 by mutating a single nonpolar amino acid, isoleucine 210 to alanine 210, in the first SET binding domain of HRX clone pCSEFAR. MUT-1 protein expressed from transiently transfected pCSMUT-1 plasmids consistently bound less SET protein (Fig. 4, lane 7) than EFAR protein expressed from pCSEFAR (Fig. 4, lane 6).

Coimmunoprecipitation Experiments Show That HRX and SET Are in a Complex with Protein Phosphatase 2A—SET has recently been shown to be a specific and noncompetitive inhibitor of PP2A (46). To determine if a phosphatase was coimmunoprecipitating with HRX, we assayed the proteins coimmunoprecipitating with myc-HRX constructs for phosphatase activity using histone H1 phosphorylated by protein kinase C as the substrate. In fact, a serine/threonine protein phosphatase activity was found coimmunoprecipitated with various tagged myc-HRX constructs (Fig. 5A). In repeated experiments, we have found that the amount of phosphatase activity in the anti-HRX coIPs roughly correlated with the amount of SET binding as determined by Western analysis. For example, HRX1385E1 and JFAR bind less phosphatase than HRX884 (Fig. 5A), and HRX1385E1 and JFAR bind less SET protein than HRX884 (Fig. 4). Consistent with these observations, we also found that protein expressed from the construct pCSMUT-1, which binds less SET (Fig. 4, lane 7) than protein from the equivalent wild-type construct pCSEFAR (Fig. 4, lane 6), binds approximately 50% less HRX-associated phosphatase activity than wild-type EFAR protein.

As shown in Fig. 5B, most of the coimmunoprecipitated phosphatase activity was sensitive to okadaic acid, a potent inhibitor of PP2A. Indeed, the main component of the coIPs has an identical okadaic acid inhibition profile as PP2A. Furthermore, Western analysis confirms the presence of PP2A in the immunoprecipitates. Western analysis with an antibody specific for the PP2A A subunit demonstrates that the PP2A A subunit is present in the immunoprecipitates (Fig. 5C). We have also found that the PP2A C subunit is present in these same immunoprecipitates (data not shown). In Fig. 5C, full-length HRX-ENL coimmunoprecipitates PP2A. We have also found that the proteins HRX884 and EFAR coimmunoprecipitate PP2A (data not shown).

DISCUSSION

In the present paper, we report the finding of a novel HRX interacting protein partner, SET. We show that the leukemic fusion protein HRX-ENL binds the SET protein. Furthermore, the HRX-SET complex appears to also include the protein PP2A. These results suggest that an HRX-SET-PP2A protein complex may be essential to the leukemogenic role of HRX fusion proteins that result from 11q23 translocations in acute leukemia. Supporting this contention is the fact that SET has been found to be a member of the SET-CAN chimeric leukemic fusion protein found in acute undifferentiated leukemia. This suggests to us the possibility that HRX fusion proteins and SET may function through a common biochemical pathway in the pathogenesis of acute leukemia, although the nature of this pathway is at present unknown. It is possible that the binding of the SET-PP2A complex by the aberrant leukemic HRX fusion proteins may trigger a normally quiescent SET-PP2A pathway.

Recently, SET has been found to be a potent inhibitor of PP2A (46). PP2A has been shown to participate in the regulation of normal cellular growth. Some transforming viruses encode proteins, including SV40 small t and polyomavirus small and middle tumor antigens, that specifically interact with PP2A and inhibit its activity (47, 48). These viral proteins are thought to participate in cellular transformation, in part, by inhibiting PP2A. Additionally, the tumor promoter okadaic acid inhibits PP2A and promotes mitosis in G2-arrested BHK1
HRX Associates with the SET Protein and PP2A

Fig. 5. A, HRX coimmunoprecipitates have phosphatase activity. Phosphatase activity assays on anti-HRX and anti-SET coIPs are shown. The coIPs and phosphatase assays are done as described under "Experimental Procedures." pCS2MT is vector only, pCS884 expresses myc-HRX884, and pCSAR expresses a full-length HRX-ENL fusion protein. pCS138SE1 and pCSFAR are HRX deletion mutants illustrated in Fig. 2. pCSSETB3 expresses a myc-SET fusion protein. B, HRX coimmunoprecipitates primarily okadaic acid-sensitive phosphatase activity similar to that of PP2A. Phosphatase inhibition studies using okadaic acid were carried out on pCSFAR (see Fig. 2) to analyze the phosphatase activity associated with pCSFAR coIPs. The HRX-associated protein phosphatase is similar to PP2A in that it is exquisitely sensitive to low concentrations of okadaic acid. C, Western analysis of HRX coimmunoprecipitates using anti-PP2A antibodies. Western blot, using anti-PP2A A-subunit antibody, of coIPs of the myc-tagged proteins expressed from the constructs listed below is shown. Lanes 1 and 2 are coIPs. Lane 3 contains PP2A A subunit as a positive control. In lane 1, pCSAR expresses a full-length HRX-ENL fusion protein, and in lane 2, pCS2MT is a vector only. PPass, protein phosphatase.

cells (49, 50). Recently, it was found that the homeobox protein HOX11 can bind and likely inhibit PP2A, resulting in the disruption of a G2/M cell-cycle checkpoint in both Xenopus oocytes and a human T-cell line (51). This alteration of the regulation of the cell cycle may be instrumental in the development of human T-cell leukemias that result from translocations that fuse the T-cell receptor α- or β-chain genes with the HOX11 gene (52, 53). An important question is whether HRX fusion proteins together with SET perform a function similar to the disruption of a G2/M cell-cycle checkpoint in both Xenopus oocytes and a human T-cell line (51). This alteration of the regulation of the cell cycle may be instrumental in the development of human T-cell leukemias that result from translocations that fuse the T-cell receptor α- or β-chain genes with the HOX11 gene (52, 53).

A clue to the mechanism of HRX fusion proteins is the finding by Rogaia et al. (54) that two different HRX fusion proteins localize to different nuclear subdomains than the wild-type HRX protein does, suggesting that part of the mechanism of activation of HRX fusion proteins may be due to altered nuclear localization. In separate studies, it has been shown that SET is a predominantly nuclear protein, and a subpopulation of PP2A is also nuclear (43, 55, 56). These studies are consistent with the formation of a nuclear HRX-SET-PP2A complex.

It is evident from Western blotting with PP2A-specific antibodies and from the studies with okadaic acid that both the PP2A catalytic subunit C and the regulatory subunit A are members of the HRX-SET-PP2A complex. What forms of PP2A are bound to HRX is an important question because PP2A core enzyme (subunits A and C) has been shown to have different substrate specificities than PP2A holoenzyme (subunits A, B, and C) (39). At present we do not actually know how PP2A is bound to the HRX-SET-PP2A complex; however, our data suggest that SET must be present for maximal PP2A binding. A serine/threonine protein phosphatase was found coimmunoprecipitated with tagged myc-SET constructs (Fig. 5A). We found that the SET-associated protein phosphatase and PP2A were inhibited by the same concentrations of okadaic acid, suggesting that the SET-associated phosphatase is PP2A (data not shown). These results suggest that PP2A is complexed to both SET and HRX primarily through its interaction with SET. Further experiments will be needed to define the contribution of HRX sequences to PP2A binding and to define the effect, if any, of HRX on the SET inhibitory effect on PP2A.

The SET binding sites on HRX overlap the A-T hooks of the A-T hooks in Mmu I(Y) have been shown to bind DNA wrapped around nucleosomes. This finding suggests that the HRX A-T hooks may also interact with nucleosomes and that SET, bound to HRX, may have a nucleosome-associated function. It is therefore interesting to note that the SET protein has homology to the nucleosome assembly protein NAP-1. Drosophila Nap-1 is a core histone shuttle that delivers histones H2A and H2B from the cytoplasm to the chromatin assembly machinery in the nucleus in a cell-cycle dependent manner. Drosophila Nap-1 is a necessary component of the reconstituted activity of ATP-facilitated assembly of extended nucleosomal arrays (57). Yeast Nap-1 proteins have nucleosome assembly activity in vitro using purified histones and relaxed plasmid DNA (58). Additionally, it has been found in vitro that yeast Nap-1 can stimulate transcription factor binding through a mechanism involving nucleosome displacement (59). That SET may have a similar in vitro function to Nap-1 is suggested by the discovery that SET is the host template activating factor 1 (TAF-1) involved in stimulating DNA replication of the adenovirus genome in a cell-free replication system, and both murine Nap-1 and yeast Nap-1 can functionally substitute for SET in this system (44).
The other A-T hook-containing proteins, HMG-I(Y) and HMGIC, did not bind SET in our coimmunoprecipitation experiments (Fig. 3B, lanes 6 and 7), suggesting that binding the SET protein is not a general feature of proteins that contain a tripeptide of A-T hooks. It is known that translocations involving the HMGIC gene result in the formation of fusion proteins that lead to the neoplastic transformation of mesenchymal tissues (35, 60). These translocations are structurally similar to HRX fusions in that the HMGIC protein is fused amino-terminal to the translocation partner, resulting in a fusion protein containing a tripeptide of A-T hooks. Recently, chromosomal rearrangements involving the HMG-I(Y) gene have also been described in mesenchymal tumors (67, 68). These data raise the possibility that the three A-T hook-containing proteins, HMG-I(Y), HMGIC and HRX, utilize similar mechanisms in the pathogenesis of mesenchymal neoplasms.

The present results further add to the contention that HRX and HRX fusion proteins may be overcoming the inhibitory effect of nucleosomes upon gene regulation by altering chromatin structure and thus transcription factor accessibility. In cells, HRX likely participates in chromatin remodeling based on analogy with the Drosophila transcriptional activator Trx. Studies in Drosophila have shown that Trx genetically opposes the action of the Polycomb group proteins that are thought to inactivate transcription through a mechanism involving modification of chromatin structure analogous to heterochromatin formation. In addition, one of the trx group members that may actually interact with Trx is Brahma, a Drosophila homologue of the yeast SWI/SNF2 protein, a component of the SWI/SNF ATP-dependent chromatin remodeling complex (61–63). Two other features of HRX are also consistent with overcoming the inhibitory effects of nucleosomes. First, HRX contains A-T hooks that may function like the hooks found in HMG-I(Y) and bind and distort nucleosomal DNA augmenting transcription. Second, SET, which binds HRX, may have functional homology to NAP and display nucleosome assembly or disassembly activity. Furthermore, at least three of the HRX fusion proteins may also be capable of modifying chromatin structure. Based on the homology with the yeast protein ANC1, it has been suggested that the HRX fusion partners ENL and AF-9 may bind the human SNF5 protein of the human SWI/SNF chromatin remodeling complex (30), and the newly described HRX-CBP fusion protein may affect chromatin structure directly through acetylation of histones (64, 65). Taken together, these data lead to the proposition that HRX fusion proteins are part of a disregulated multi-subunit chromatin remodeling complex that leads to leukemogenesis through inappropriate transcriptional control of target genes.

Acknowledgments—We thank Dan Wu for helpful discussions, Marc Mumbry for purified PP2A, Terry Copeland for anti-SET antibodies, Jim Roberts for anti-nc monoclonal 9E10, Boguslaw Kwiatkowski for help with two-hybrid analysis, Zahi Damuni for advice on phosphate assays, Raymond Reeves for the HMG-I(Y) cDNA, K. Chada for the chromatin remodeling complex (31), and the newly described HRX-CBP fusion protein may affect chromatin structure directly through acetylation of histones (64, 65). Taken together, these data lead to the proposition that HRX fusion proteins are part of a disregulated multi-subunit chromatin remodeling complex that leads to leukemogenesis through inappropriate transcriptional control of target genes.

REFERENCES

1. Tkachuk, D. C., Kohler, S., and Cleary, M. L. (1992) Cell 71, 691–700
2. Hunger, S. P., Tkachuk, D. C., Amylon, M. D., Link, M. P., Carroll, A. J., Welborn, J. L., Willman, C. L., and Cleary, M. L. (1993) Blood 81, 3177–3183
3. Djabali, M., Selleri, L., Parry, P., Bower, M., Young, B. D., and Evans, G. A. (1992) Nat. Genet. 2, 113–118
4. Yu, B. D., Hess, J. L., Horning, S. E., Brown, G. A., and Korsmeyer, S. J. (1993) Nature 365, 505–508
5. Breen, T. R., and Harte, P. J. (1993) Development 119, 139–144
6. Yu, B. D., Hess, J. L., Horning, S. E., Brown, G. A., and Korsmeyer, S. J. (1993) Nature 365, 505–508
7. Li, M., Makojine, A., and Damuni, Z. Z. (1996) J. Biol. Chem. 271, 11059–11062
8. Dalla, D., Chahar, L. K., Martin, B. L., Jaspers, S., Miller, T. B., Brautigan, D. L., and Roberts, T. M. (1993) Cell 70, 167–176
9. Warner, G., Ruediger, R., Slaughter, C., and Munby, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8900–8904
10. Hof, S., and Song, O. (1989) Nature 340, 245–246
11. Kremmer, E., Ohst, K., Kiefer, J., Brewis, N., and Walter, G. (1997) Mol. Biol. Chem. 66, 2598–2601
12. Kremmer, E., Loh, H. S., and Cleary, M. L. (1995) Biochemistry 35, 149–153
13. Leshkowitz, D., Rozenblatt, O., Nakamura, T., Yano, T., Dautry, V., Croce, C. M., and Seto, M. (1996) Cancer Res. 56, 1766–1769
14. Joh, T., Kagami, Y., Yamamoto, K., Segawa, T., Takahashi, J., Takahashi, T., Ueda, R., and Seto, M. (1996) Oncogene 13, 1945–1953
15. Ashar, H. R., Fejzo, M. S., Tkachenko, A., Zhou, X., Fletcher, J. A., Okuda, A., Kikuchi, A., and Matsumoto, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4729–4733
16. So, C. W., Caldas, C., Liu, M. M., Chen, S. J., Huang, Q. H., Gu, L. J., Sham, S. C. W., Caldas, C., Liu, M. M., Chen, S. J., Huang, Q. H., Gu, L. J., and Seto, M. (1996) Oncogene 13, 1945–1953
17. Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jenkins, N. A., and Copeland, N. G. (1996) Genet. 12, 149–153
18. Loh, R., and Wolffe, A. P. (1996) Biochemistry 35, 5063–5074
19. Workman, J. L., and Kingston, R. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 90, 10610–10614
20. Broeker, P. L., Harden, A., Rowley, J. D., and Zeleznik, L. N. (1996) Curr. Top. Microbiol. Immunol. 215, 259–268
21. Joh, T., Kagami, Y., Yamamoto, K., Segawa, T., Takahashi, J., Takahashi, T., Ueda, R., and Seto, M. (1996) Oncogene 13, 1945–1953
22. Yu, B. D., Hess, J. L., Horning, S. E., Brown, G. A., and Korsmeyer, S. J. (1993) Cell 71, 691–700
23. Turowski, P., Fernandez, A., Favre, B., Lamb, N. J., and Hemmings, B. A. (1995) J. Biol. Chem. 270, 2563–2568
24. Fornerod, M., Boer, J., van Baal, S., Jaegle, M., von Lindern, M., Murti, K. G., and Copeland, N. G. (1996) J. Biol. Chem. 271, 1739–1748
25. Broeker, P. L., Harden, A., Rowley, J. D., and Zeleznik, L. N. (1996) Curr. Top. Microbiol. Immunol. 215, 259–268
26. Bitescu, M., Selleri, L., Parry, P., Bower, M., Young, B. D., and Evans, G. A. (1992) Nat. Genet. 2, 113–118
27. Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jenkins, N. A., and Copeland, N. G. (1996) Genet. 12, 149–153
28. Krenn, J. T., Kagami, Y., Yamamoto, K., Segawa, T., Takizawa, J., Takahashi, T., Ueda, R., and Seto, M. (1996) Oncogene 13, 1945–1953
29. Ishimi, Y., Yasuda, H., Hirosumi, J., Hanaoka, F., and Yamada, M. (1983) J. Biochem. (Tokyo) 94, 735–744
30. Takeda, K., Nakamura, T., and Nakamura, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8900–8904
31. Robert, A., Suraud, E., and Rabbitts, T. H. (1993) Nature 365, 505–508
32. Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jenkins, N. A., and Copeland, N. G. (1996) Genet. 12, 149–153
33. Kremmer, E., Ohst, K., Kiefer, J., Brewis, N., and Walter, G. (1997) Mol. Cell. Biol. 17, 1692–1701
34. Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jenkins, N. A., and Copeland, N. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5758–5762
59. Walter, P. P., Owen-Hughes, T. A., Cote, J., and Workman, J. L. (1995) Mol.
Cell. Biol. 15, 6178–6187
60. Schoenmakers, E. F., Wanschura, S., Mols, R., Bailerdisch, J., Van den Berghe,
and Van de Ven, W. J. (1995) Nat. Genet. 10, 436–444
61. Burns, L. G., and Peterson, C. L. (1997) Biochim. Biophys. Acta 1350, 159–168
62. Dingwall, A. K., Beek, S. J., McCallum, C. M., Tamkun, J. W., Kalpana, G. V.,
Geff, S. P., and Scott, M. P. (1995) Mol. Biol. Cell 6, 777–791
63. Tamkun, J. W. (1995) Curr. Opin. Genet. Dev. 5, 473–477
64. Taki, T., Sako, M., Tsuchida, M., and Hayashi, Y. (1997) Blood 89, 3945–3950
65. Sobulo, O. M., Borrow, J., Tomek, R., Reshmi, S., Harden, A., Schlegelberger,
B., Housman, D., Doggett, N. A., Rowley, J. D., and Zeleznik-Le, N. J. (1997)
Proc. Natl. Acad. Sci. U. S. A. 94, 8732–8737
66. Lavau, C., Szilvassy, S. L., Slany, R., and Cleary, M. L. (1997) EMBO J. 16,
4226–4237
67. Williams, A. J., Powell, W. L., Collins, T., and Morton, C. C. (1997) Am. J.
Pathol. 150, 911–918
68. Tkachenko, A., Asrar, H. R., Meleni, A. M., Sandberg, A. A., and Chada, K. K.
(1997) Cancer Res. 57, 2276–2280