INTERACTIONS BETWEEN TWO
CAENORHABDITIS ELEGANS SEX DETERMINING
PROTEINS: HER-1 AND TRA-2A

by

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A Thesis submitted in conformity with the requirements for the degree of
Master of Science
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Abstract

Interactions between two *Caenorhabditis elegans* sex determining proteins, HER-1 and TRA-2A. Degree of Master of Science, 2000. Roxana Sultan. Graduate Department of Molecular and Medical Genetics, University of Toronto.

The free-living soil nematode *Caenorhabditis elegans* exists in nature as one of two possible sexes, the male or the self-fertile hermaphrodite. Most genes controlling sex determination in *C. elegans* have been cloned and sequenced. These genes have been ordered into a signal transduction cascade based on their epistatic interactions.

I have uncovered evidence supporting a direct interaction between the proteins encoded by two of the genes in the pathway, *her-1* and *tra-2*. This was achieved through binding assays involving immunofluorescent and immunoblotting techniques. The proposed interaction sheds light on the possible mechanisms by which HER-1 negatively regulates the activity of TRA-2A in male development.
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LIST OF ABBREVIATIONS AND GENE NAMES

\( a \) : anti

BSA : bovine serum albumin

C-terminus : carboxy terminus

CMV : cytomegalovirus

DAPI : 4', 6'-diamidino-2-phenylindole

dpy : dumpy

DTT : dithiothreitol

EDTA : ethylenediamine tetra-acetate

EF : elongation factor

eg : enhanced gain of function

egl : egg-laying defective

EGTA : ethyleneglycol-bis-[\( \beta \)-aminoethyl ether]-N, N, N, N’-tetraacetic acid

fem : feminization

FITC : fluorescein isothiocyanate

fog : feminization of germline

fox : feminizing locus on X

gf : gain of function

GFP : green fluorescent protein

HA : hemaglutinin

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

her : hermaphroditization

kb : kilobase pairs

kDa : kilo Daltons

lf : loss of function

mab : male abnormal

Mb : megabase

MBP : maltose binding protein

mog : masculinization of germline

MOI : multiplicity of infection

MTPBS : mouse tonicity phosphate buffered saline

N-terminus : amino terminus

NHR : nuclear hormone receptor

PBS : phosphate buffered saline

PD : proportionate distance

pfu : plaque forming unit
PMSF: phenylmethanesulphonyl fluoride
RNP: ribonucleioprotein
sdc: sex and dosage compensation
SDS: sodium dodecyl sulfate
SDS-PAGE: SDS-polyacrylamide gel electrophoresis
sex: signal element on X
SMC: structural maintenance of chromosomes
TBST: Tris-buffered saline + Tween-20
TCID₃₀: tissue culture infectious dose
tra: transformer
xol: XO lethal
1. Introduction

1.1 General Introduction

The study of developmental biology addresses the fundamental question of how cells adopt and maintain specific fates in eukaryotic organisms. The manner in which cells establish themselves as one of two dimorphic sexes is essentially a subset of these processes. To explore the molecular mechanisms underlying these regulatory events, the processes of cell-to-cell communication and signal transduction can be examined. To transmit signals specifying developmental fate, cells sense and process stimuli through molecular circuits consisting of receptors, ligands, channels and regulatory proteins. A protein signal secreted from a signaling cell can be received by a neighboring target cell via binding of the ligand to a transmembrane receptor protein. This initial interaction can induce subsequent amplification and transmission of the external signal through the activation of a signal transduction cascade within the target cell, which in turn can specify its fate. These signaling systems are the molecular basis for cell-cell inductive interactions and can provide a mechanism by which cells can adopt and commit to a specific sexual fate, that is, sex determination. Since sex determination is ubiquitous across many phyla, analysis of the genetic and molecular mechanisms of cellular sexual fate determination is an area of interest for molecular, evolutionary and developmental biologists alike.
1.2 *C. elegans* as a Model Organism

The nematode *C. elegans* possesses a number of characteristics which make it useful as a model for the study of sex determination. At an average size of 1.5 mm and a life cycle of approximately three days at 20°C, the culture and maintenance of *C. elegans* is convenient and inexpensive.

More specifically:

1. The transparency of the worm allows direct examination of its internal structures.
2. The *C. elegans* genome is small - about 100 Mb carried on five autosomes and one sex chromosome - and has been completely sequenced, allowing analysis of genome organization and evolutionary divergence between species.
3. *C. elegans* has a small number of somatic cells - 959 in the hermaphrodite and 1031 in the male - yet possesses most of the organ structures of more complex organisms.
4. The pattern and number of cell divisions from fertilization to adulthood (cell lineage) has been found to be invariant between individuals, permitting the examination of cell fate decisions by genetic methods (Sulston and Horvitz, 1977; Sulston *et al.*, 1983).
5. Hermaphroditic reproduction by self-fertilization permits maintenance of mutations which may inhibit mating.
6. Microinjection of DNA into hermaphrodite gonads enables establishment of transgenic lines expressing genes of interest, which can be further examined by techniques such as *in situ* hybridization and antibody staining.
Taken together, these qualities make *C. elegans* a convenient and useful model organism for the study of many aspects of biology.

### 1.3 Sexual Dimorphism in *C. elegans*

*C. elegans* animals exist in nature as either males or self-fertilizing hermaphrodites (Figure 1). As reviewed by Hodgkin (1988) sexual dimorphism exists in most of the tissues and structures of *C. elegans* including the intestine, germline, musculature, nervous system, hypodermis, and gonad. The hermaphrodite is somatically female but its germline produces first sperm and then oocytes over the course of development. The additional cells in the male as compared to the hermaphrodite are mainly located in its complex tail, which consists of a copulatory bursa arranged in a cuticular fan, sensory rays, spicules and a cloaca. The male tail is necessary for mating with hermaphrodites. The hermaphrodite tail tapers into a simple spike. The hermaphroditic gonad is a bilobed structure consisting of ovaries, spermatheca and a uterus. The male gonad is uni-lobed and consists of a seminal vesicle, testis and vas deferens. Given this extensive sexual dimorphism, which affords many ways to assay fate choices, sex determination clearly has global effects on *C. elegans* development.

### 1.4 The X:A Ratio and Dosage Compensation

The primary determinant of phenotypic sex in *C. elegans* is the ratio of X chromosomes to complete sets of autosomes (Madl and Herman, 1979). XX embryos develop as hermaphrodites whereas XO animals develop as males. Polyploids with an X:A < 0.67 develop as male whereas those with ratios > 0.75 are hermaphrodites. Quantification of
the X:A ratio may require the existence of both "numerator" and "denominator" elements to permit counting of the X chromosomes and autosomes, respectively. At least two genes on the X chromosome are involved in the mechanism of X chromosome counting and are therefore candidate "numerator" elements - sex-1 (single element on X) and fox-1 (feminization locus on X) (Carmi et al., 1998; Hodgkin et al., 1994; Nicoll et al., 1997; Skipper et al., 1999). The proposed activities of sex-1 and fox-1 will be discussed below.

As yet, no autosomal denominator elements have been identified.

One consequence of such a chromosome counting mechanism is a differential dose of X chromosomes between the hermaphrodite and male, wherein X-linked genes are present at twice the copy number in hermaphrodites as compared to males. To equalize X-linked gene expression, a subset of genes in the sex determination pathway, known as the sdc genes, acts to regulate effective sex chromosome dosage in a process known as dosage compensation, by reducing the level of transcription from both hermaphroditic X chromosomes (Meyer and Casson, 1986).

The X:A ratio acts as the initial level of control in C. elegans sexual differentiation and specifies the activity or repression of a defined cascade of genes governing dosage compensation and somatic and germline sex determination. Epistatic analysis of the loss of function alleles of genes involved in sex determination has resulted in the proposal of a regulatory cascade (Figure 2) which outlines a series of genetic interactions (Doniach and Hodgkin, 1984; Hodgkin, 1980; Hodgkin, 1986; Miller et al., 1988; Villeneuve and
Meyer, 1987). However, the specific mechanisms of regulation on a molecular level are not necessarily defined by the genetic pathway.

Immediately downstream of the X:A signal is the xol-1 (XO-lethal) gene. Activity of xol-1 appears to ensure that down-regulation of X-linked transcription does not occur in XO animals as it does in XX animals (Miller et al., 1988). Loss of function mutations in xol-1 are karyotype-specific and found to be lethal to XO animals as activity of xol-1 prevents the single X in XO males from being dosage compensated. Any surviving xol-1 XO animals are feminized. Loss of function mutations in xol-1 have no affect on XX development, but overexpression of xol-1 in XX animals results in lethality and masculinization of survivors (Rhind et al., 1995).

The aforementioned sex-1 and fox-1 have been implicated in the regulation of xol-1. FOX-1 has an RNP type RNA-binding domain (Nicoll et al., 1997; Skipper et al., 1999) which suggests that it may regulate the xol-1 gene at the translational level. The SEX-1 protein belongs to the nuclear hormone receptor (NHR) superfamily and has been shown to bind the xol-1 promoter in vitro and in vivo in a transgenic experiment (Carmi et al., 1998), suggesting that SEX-1 likely represses xol-1 transcription directly.

The xol-1 gene negatively regulates the sdc-1, sdc-2 and sdc-3 (sex and dosage compensation) genes. Rhind et al. (1995) showed that transgenic arrays carrying a non-functional version of sdc-2 suppressed the xol-1(gf) phenotype. This suggests that sdc-2 may be the direct target of XOL-1 activity. Loss of function alleles of the sdc genes cause
lethality and strong masculinization in XX animals as a result of altered dosage compensation, but have no affect on XO development (Nusbaum and Meyer, 1989).

While the xol-1 and sdc genes coordinately control sex determination and dosage compensation, the genes downstream in the pathway govern either dosage compensation or sex determination alone. Activity of the sdc genes stimulates the activation of a set of autosomal genes known as the dpy (dosage compensation dumpy) genes - dpy-21, dpy-26, dpy-27 and dpy-28 (Hodgkin, 1983; Hsu and Meyer, 1994; Meyer and Casson, 1986). As reviewed in Villeneuve and Meyer (1990), loss of function mutations in these dpy genes lead to increased expression of X-linked genes resulting in lethality or a stout body (dumpy) phenotype in XX animals.

Evidence has suggested that the SDC proteins form a complex with some components of the dpy branch of the pathway to bind the X chromosomes of XX animals and facilitate dosage compensation (Davis and Meyer, 1997; Dawes et al., 1999). DPY-26 and DPY-27 have been shown to interact specifically with the X chromosome in XX embryos and the interaction is contingent upon SDC-2 and SDC-3 (Chuang et al., 1994; Lieb et al., 1996). DPY-27 belongs to the SMC (structural maintenance of chromosomes) family of proteins which in yeast and Xenopus are involved in chromosomal condensation. This suggests that DPY-27’s role in dosage compensation may involve X chromosome down-regulation via condensation (Chuang et al., 1994).
1.5 Somatic Sex Determination: HER-1A

The pathway regulating sex determination downstream of the sdc genes consists of seven genes. Analysis of the epistatic interactions between the recessive alleles of these genes suggested a hierarchical set of interactions which have been ordered into a negative regulatory pathway (Hodgkin, 1980; Hodgkin, 1986). Mutations in these genes result in transformations of sexual fate which can override the primary X:A signal.

Once the X:A signal has been interpreted by the sdc genes, the signal is conveyed to the her-1 (hermaphroditization) gene, the first in the cascade specific to sex determination (Figure 2). Activity of the her-1 gene is both necessary and sufficient for specification of male fate. Recessive her-1 loss-of-function mutations that knock out gene function cause XO animals to develop as phenotypic hermaphrodites, but have no influence on XX development (Hodgkin, 1980). Conversely, the semi-dominant gain of function alleles, *m695* and *y101*, cause overexpression of the gene and variably masculinize XX animals (Perry *et al*., 1994; Trent *et al*., 1988). Since the loss of function phenotype is opposite to that of the gain-of-function allele, her-1 can be characterized as a genetic switch.

The her-1 gene produces two male-specific transcripts from two tandem promoters, P1 and P2: the relatively rare 1.2 kb *her-1a*, and the more abundantly expressed 0.8 kb *her-1b* (Figure 3) (Trent *et al*., 1991). Both transcripts are present at much higher levels in XO animals as compared to XX, but only the longer of the two appears to be necessary for male development (Perry *et al*., 1993).
The two gain-of-function alleles map to the P1 promoter and may delineate a potential site for interaction with repressors that are active in XX animals (Perry et al., 1994; Trent et al., 1988; Trent et al., 1991). Since XX animals with loss-of-function mutations in sdc-1, sdc-2 or sdc-3 express her-1 transcripts (DeLong et al., 1993; Trent et al., 1991) it was suggested that her-1 expression may be controlled at a transcriptional level by one or more of the sdc genes. Schauer and Wood (1990) used run-on transcription assays to demonstrate that the regulation of her-1 does indeed take place at the transcriptional level. Further, Dawes et al. (1999) have shown that SDC-2 binds portions of her-1 in vivo.

Perry et al. (1993) showed that her-1a encodes a novel 175 amino acid, cysteine-rich protein, HER-1A, which is approximately 20 kDa. The her-1b gene product, whose function is as yet unknown, consists of the 64 C-terminal amino acids of HER-1A. The HER-1A protein has a secretion signal sequence at its N-terminus which is required for its activity. Hunter and Wood (1992) demonstrated through mosaic analysis that her-1 functions in a non-cell-autonomous fashion. That is, her-1(-) cells can be recruited to the male sexual fate when developing in the presence of her-1(+) cells. Furthermore, her-1(-) cells can be feminized when developing in a field of her-1(-) cells.

Taken together, these data suggest that HER-1A could function as a signaling ligand. In C. elegans development, most of the sexual structures are formed by the descendants of more than one precursor cell, so cell-to-cell communication could be a mechanism by which sexual fate is adopted and maintained globally. Cells developing in the proximity of
one another may be destined to be components of a particular structure as prescribed by
the cell lineage, but they must also be coordinated to attain the same sexual fate. If HER-
1A functions as a ligand, then there must be a corresponding receptor with which it
interacts to promote the male developmental program. The proximal genetic target of her-
1 is tra-2, making it a suitable candidate to encode such a protein.

1.6 Somatic Sex Determination: TRA-2A

Loss of function mutations in the tra-2 (transformer) gene cause XX animals to develop
as non-mating pseudomales, but have no affect on XO development (Hodgkin and
Brenner, 1977). On the other hand, gain of function alleles cause XX animals to develop a
completely feminized germline (true females) and XO animals to become mildly feminized
(Doniach, 1986, Goodwin et al., 1993; Schedl and Kimble, 1988). Thus, it would appear
that an activity conferred by tra-2 is involved in somatic feminization. Three different
transcripts have been detected from the tra-2 gene: a 1.8 kb mRNA in the hermaphrodite
germline, a 1.9 kb transcript in larval and adult males, and a 4.7 kb mRNA which is
expressed in both sexes and found to encode a novel 170 kDa protein with nine
hydrophobic domains, referred to as TRA-2A (Kuwabara et al., 1992; Kuwabara et al.,
(1992) predicted that the N-terminal portion of the 170 kDa protein is extracellular while
the acidic C-terminal end is proposed to be intracellular.

The 1.8 kb transcript has been found to encode the C-terminal intracellular portion of
TRA-2A and is known as TRA-2B. It has been shown that the evolutionarily divergent
nematode species *Caenorhabditis briggsae* also has a *tra-2* gene but expresses only a 4.7 kb transcript, which suggests that this mRNA accounts for most of *tra-2*’s activity *in vivo* (Kuwabara, 1996).

As suggested by the epistatic relationship between *her-1* and *tra-2* as well as the fact that the *her-1(*ff*) phenotype is opposite to that of the *tra-2(*ff*)*, there may be an antagonistic relationship between the activities of *her-1* and *tra-2*. It is possible that the relative levels of HER-1 and TRA-2 affect the sexual fate decision, as demonstrated by the feminization of XO animals in which overexpression of *tra-2* has been engineered (Kuwabara and Kimble, 1995). Furthermore, a point mutation in the extracellular domain of TRA-2A (R177K missense) known as the *tra-2* (*eg*) allele for enhanced gain of function, has been shown to have the same phenotype as a *her-1(*ff*) mutation and is insensitive to negative regulation by *her-1(*gf*) mutations (Hodgkin and Albertson, 1995; Kuwabara, 1996). The *tra-2*(eg) mutation may implicate the arginine residue as a potential regulatory site on TRA-2A, and perhaps delineate a site of contact with a negative regulator. Taken together, these data provide evidence supporting the hypothesis that TRA-2A may be the direct target of HER-1’s masculinizing activity.

The expression of *tra-2* appears to be related to the phenotypic sex of the organism and not the X:A karyotype. It has been shown that the 4.7 kb transcript is expressed at fifteen fold higher levels in the hermaphrodite as compared to the male (Okkema and Kimble, 1991). This would suggest some sort of feedback communication between *tra-2* and *tra-1*, the terminal regulatory gene in the sex determination pathway. Graves *et al.* (1999)
showed that the 3' untranslated region of the *tra-2* transcript can be bound by the product of the *tra-l* gene, TRA-1A and that this is necessary for the export of *tra-2* mRNA to the cytoplasm. This suggests that the terminal regulator plays a role in the translational control of *tra-2* expression.

Kuwabara and Kimble (1995) showed that increased expression of the C-terminal portion of TRA-2A feminizes XX *tra-2(If)* animals as well as XO *tra-2(-)*) animals. This suggests that the intracellular portion of the protein is involved in *tra-2*'s feminizing activity. Given that *tra-2* is hypostatic to the *fem* genes in the sex determination pathway and these *fem* genes are required for male development, the product(s) of *fem* expression represent suitable candidates for regulatory targets of the C-terminus of *tra-2*. Mehra et al. (1999) presented evidence that the TRA-2B protein interacts directly with the FEM-3 protein to negatively regulate its activity. This presents the possibility that the increased expression of *tra-2* in wildtype XX animals serves to titrate one or more of the FEM proteins. Activity of the FEM proteins is required for the adoption of male fate and will be discussed below.

### 1.7 Somatic Sex Determination: TRA-3

Null mutations in the *tra-3* (*transformer*) gene behave like weak *tra-2* loss-of-function alleles in that they cause XX animals to develop as incomplete males (Hodgkin and Brenner, 1977). However this effect can be maternally rescued. It has been suggested that *tra-3* may act as a positive regulator of *tra-2*, as demonstrated by the fact that *tra-2(If)*; *tra-3(If)* double mutants have essentially the same phenotype as *tra-2(If)* single mutants
and that $tra-3(II)$ can be suppressed by strong, hypermorphic $tra-2(gf)$ alleles (Doniach, 1986).

The 648 amino acid protein encoded by $tra-3$ shows homology to the large subunit of the calpain family of proteases (Barnes and Hodgkin, 1996) but lacks the characteristic calcium-binding EF domain of these proteases. Sokol and Kuwabara (2000) have presented evidence that despite the absence of EF hands, TRA-3 has calcium-dependent proteolytic activity, with TRA-2A as one of its substrates. Further, they have demonstrated that the cleavage of TRA-2A generates a peptide which is predicted to have feminizing activity. They also showed that TRA-3 undergoes calcium-mediated autolysis and that the feminizing activity of $tra-3$ is dependent on $tra-2$ in vivo.

1.8 Somatic Sex Determination: FEM-1

The $fem$ (feminization) genes promote spermatogenesis in males and hermaphrodites and appear to be essential for male somatic development. They are targets of negative regulation by $tra-2$ and $tra-3$ and down-regulate $tra-1$ in the male soma (Doniach and Hodgkin, 1984; Hodgkin, 1986; Kimble et al., 1984). Loss of function mutations in the $fem$ genes cause XX and XO animals to become feminized and all three have been shown to have maternal effects.

Doniach and Hodgkin (1984) showed that homozygous $fem-1 (m-z-)$ XX and XO animals develop as true females. Further, it was shown that twenty percent of XX homozygous $fem-1 (m-z-)$ animals, which are the progeny of $fem-1$ heterozygous hermaphrodites,
develop as self-fertile hermaphrodites which in turn produce completely female broods. XO animals of the same genotype develop as non-mating pseudomales with incompletely masculinized tails.

The *fem-1* transcript and translational product are expressed throughout development in both sexes, yet the hermaphroditic somatic tissues are not masculinized by its presence (Gaudet *et al.*, 1996). This would suggest that FEM-1 is modulated post-transcriptionally. The protein itself consists of 656 amino acids and is a soluble, intracellular molecule with seven ankyrin repeats at its N-terminus (Gaudet *et al.*, 1996; Spence *et al.*, 1990). Several *fem-1* mutations map to this ankyrin repeat region (Chin-Sang, 1998) and such domains have been shown to mediate protein-protein interactions in other proteins (Bennett, 1992; Michaely and Bennett, 1992).

1.9 Somatic Sex Determination: FEM-2

The *fem-2* gene demonstrates complete maternal rescue in that the presence of maternal *fem-2* in the oocyte is sufficient to promote spermatogenesis in XX animals and somatic masculinization of XO animals which are *fem-2(-)* at 25° C (Hodgkin, 1986; Kimble *et al.*, 1984). A temperature-sensitive effect of *fem-2* was demonstrated by the incomplete feminization of *fem-2 (m-z-)* XO animals at 15° C but their full feminization at 25° C (Hodgkin, 1986; Pilgrim *et al.*, 1995). This presents the possibility that there may be some functional redundancy between the *fem* activities which can partially compensate for the absence of *fem-2* at permissive temperatures.
The *fem-2* mRNA has been found to be expressed at all stages of development but is particularly abundant in XX animals during oogenesis, and codes for a 449 amino acid protein with homology to type 2C serine/threonine protein phosphatases (Pilgrim *et al.*, 1995). It was shown by Chin-Sang and Spence (1996) that FEM-2's enzymatic activity is necessary for male development and that it physically interacts with FEM-3 *in vitro*. This suggests that FEM-3 may be a dephosphorylation target of FEM-2.

1.10 Somatic Sex Determination: FEM-3

The activity of *fem-3* seems to be the limiting factor with respect to the *fem* genes' masculinizing activity as it appears to behave in a dose-sensitive manner. Loss-of-function mutations in *fem-3* cause both XX and XO animals to develop as females and this effect demonstrates a weak haploinsufficiency in that five to ten percent of the XX *(m-2-)* progeny of *fem-3*-* animals are female even if the mothers have contributed a wildtype copy of *fem-3* (Barton *et al.*, 1987; Hodgkin, 1986). Furthermore, the maternal effects are demonstrated in that heterozygous progeny of *fem-3*-* *fem-3* mothers, which would be *fem-3*-* (m-2-), show some germline feminization in XX animals and both germline and somatic feminization of XO animals (Hodgkin, 1986).

XX animals carrying gain-of-function mutations in the 3' untranslated region of *fem-3* resulting in increased protein production make only sperm, which is characterized as a Mog (*masculinization of germline*) phenotype (Barton *et al.*, 1987). Thus, *fem-3* regulation is instrumental in ensuring that the switch from spermatogenesis to oogenesis
takes place in XX animals. Mehra et al. (1999) showed that overexpression of \textit{fem-3} in XX animals causes them to develop as non-mating pseudomales. Interestingly, \textit{fem-3} gain of function mutations can partially rescue male somatic development in XO \textit{tra-1(gf)} animals (Schedl et al., 1989).

The \textit{fem-3} gene was found to encode a novel 388 amino acid protein (Ahringer et al., 1992). The FEM-3 protein interacts \textit{in vitro} with both TRA-2 and FEM-2, and the relative levels of TRA-2 and FEM-3 affect the sexual fate decision (Chin-Sang and Spence, 1996; Mehra et al., 1999). Therefore, TRA-2 and FEM-3 can both be viewed as molecular switches in the sexual fate decision-making process.

1.11 Somatic Sex Determination: TRA-1

The terminal regulator in the \textit{C. elegans} sex determination pathway is the \textit{tra-1} (\textit{transformer}) gene, which is epistatic to all the other components in the cascade and is both necessary and sufficient for directing female somatic development. Loss of function mutations in \textit{tra-1} cause XX animals to become somatically masculinized (Hodgkin and Brenner, 1977; Hodgkin, 1987; Schedl et al., 1989). Dominant gain of function alleles of \textit{tra-1} cause both XO and XX animals to develop as females (Hodgkin, 1987) and these mutations map to the N-terminus of the TRA-1 protein (de Bono et al., 1995). This site therefore delineates a possible site of regulation. It was shown by Zarkower and Hodgkin (1992) that \textit{tra-1} transcripts are expressed at equivalent proportions in wildtype XX and XO animals, suggesting that the down-regulation of \textit{tra-1} in XO animals occurs post-transcriptionally.
Alternative splicing results in the generation of two tra-1 products, TRA-1A and TRA-1B. The TRA-1A protein is composed of 1110 amino acids and carries five zinc finger motifs, while TRA-1B consists of the N-terminal 288 amino acids and two zinc fingers of TRA-1A (Zarkower and Hodgkin, 1992). TRA-1A, but not TRA-1B, has been shown to bind sequence-specifically to DNA in vitro (Zarkower and Hodgkin, 1993), which presents the possibility that it may act as a transcription factor involved in the activation of transcription of hermaphrodite-specific genes and/or repression of male-specific gene expression. Conradt and Horvitz (1999) and Raymond et al. (1998) have presented evidence that the genes mab-3 and egl-1 are likely direct targets of TRA-1A-mediated repression in hermaphrodites.

1.12 Molecular Model of Interactions

The transmission of a message from the membrane to the nucleus can involve a series of protein-protein contacts. A molecular model of interactions has been suggested based on the interactions between the genes in the signal transduction pathway (Figure 4). As noted earlier, the high X:A ratio in XX animals signals the sdc genes to repress transcription of her-1 whereas in XO animals the sdc genes are inactive and therefore transcription of her-1 is permitted. It is thus proposed that binding of the secreted HER-1A ligand to the membrane-bound receptor TRA-2A provides the stimulus for male development. Further, it is suggested that in the XX animal, FEM-3 is sequestered by TRA-2A and TRA-1A is permitted to direct transcription of hermaphrodite-specific genes. Since FEM-3 is the limiting component of fem activity and all three fem products are required for male
development, it is believed that titration of FEM-3 should be sufficient to block the male program.

In the XO animal, the extracellular interaction between HER-1A and TRA-2A is thought to induce a conformation in TRA-2A which inhibits its ability to inactivate FEM-3 intracellularly, leaving the FEM proteins free to down-regulate TRA-1A and promote male development. The evidence for a FEM-2/FEM-3 complex suggests that FEM-3 may be involved in either activating the FEM-2 phosphatase or in directing it to its substrate. One possible substrate is TRA-1A, as it is the genetic target of $fem$ activity.

1.13 Germline Sex Determination

The developmental progress of XX hermaphrodites presents somewhat of a paradox in that transient upregulation of $fem-3$ and down-regulation of $tra-2$ must take place to permit spermatogenesis, which occurs during the third and fourth larval molts. This presents the possibility that the activity of genes controlling sex determination must be regulated differently in the germline than in the soma. An overview is shown in Figure 5.

In contrast to the soma, where $tra-1$ is the terminal regulator, the $fem$ genes appear to be epistatic to $tra-1$ in the germline. It has been shown that $fem(lf); tra-1(lf)$ double mutants are somatically male but produce only oocytes (Doniach and Hodgkin, 1984; Hodgkin, 1986). XX animals with $tra-1(lf)$ mutations produce sperm but perform limited oogenesis as well, while $tra-1(lf)$ XO animals produce much lower quantities of sperm as compared to the wild type (Hodgkin, 1987; Schedl et al., 1989). Taken together, these results imply
that the activity of \textit{tra-1} is required in \textit{XX} animals for the upregulation of oogenesis and down-regulation of spermatogenesis, while in \textit{XO} animals it is necessary for the maintenance of spermatogenesis and inhibition of oogenesis. Cline and Meyer (1996) hypothesized that the interaction of \textit{fem} and \textit{tra-1} gene products in \textit{XO} and larval \textit{XX} animals promotes spermatogenesis and prevents oogenesis, but in the absence of \textit{fem} activity, as is the case in adult \textit{XX} animals, the reverse is true.

The \textit{fog} and \textit{mog} genes have been found to exert effects solely in the germline. Loss of function alleles in \textit{fog-1} cause both \textit{XX} and \textit{XO} animals to develop normally in the soma but these animals fail to produce sperm in the germline, suggesting that activity of this gene is required for spermatogenesis in both sexes (Barton and Kimble, 1990; Ellis and Kimble, 1995). On the other hand, \textit{fog-2(lf)} mutations cause \textit{XO} animals to develop normally but \textit{XX} animals do not undergo spermatogenesis (Schedl and Kimble, 1988). This implies that \textit{fog-2} activity is involved in hermaphroditic spermatogenesis and would thus need to be switched off to permit subsequent oogenesis. Further, Schedl and Kimble (1988) showed that \textit{tra-2(lf); fog-2(lf)} double mutant \textit{XX} animals express the \textit{tra-2(lf)} phenotype which suggests that \textit{tra-2} is epistatic to \textit{fog-2} in the \textit{XX} germline.

Loss of function alleles of \textit{mog-1} cause \textit{XX} animals to perform spermatogenesis continuously and fail to produce oocytes, while \textit{mog-1(lf); fem(lf)} perform oogenesis (Graham and Kimble, 1993; Graham et al., 1993). This suggests that \textit{mog-1} activity may be involved in initiating the switch from spermatogenesis to oogenesis in the hermaphrodite. The epistasis of \textit{fem} and \textit{fog} mutations to those of \textit{mog} suggests that
wildtype mog activity negatively regulates one or more of these upon completion of spermatogenesis in hermaphrodites.

1.14 Thesis Overview

The detailed molecular interactions underlying the genetic model presented in Figure 5 are not completely known. One aspect of interest is whether the genetic interactions between the C. elegans sex determination genes her-1 and tra-2 result from a direct physical interaction. This thesis endeavors to present evidence supporting the existence of a direct interaction between the proteins HER-1A and TRA-2A, using immunoblotting and immunofluorescence techniques.
2. Materials and Methods

2.1 Anti-HER-1 Antibody Purification

I purified α HER-1 antibodies from crude rat serum by using affinity chromatography with a maltose-binding protein/HER-1 fusion as ligand. The rats had been immunized with a bacterially-expressed histidine-tagged HER-1 fusion protein (N. Rajwans, personal communication). These antibodies were used in subsequent experiments to detect HER-1.

Maltose Binding Protein HER-1 Fusion Purification:

To prepare MBP-HER-1 for use in purification of α-HER-1 antibodies, competent bacterial BL21 cells were transformed with the plasmid pMPJ2-443. This plasmid contains a gene encoding a maltose-binding protein fused to HER-1, under control of the P_{lac} promoter. Two 250 mL cultures of these cells were grown overnight and expression of the MBP-HER-1 fusion was induced. The cells were then harvested, sonicated and the cellular debris centrifuged out. Five mL of the crude extract was passed over a 1 mL amylose/agarose column (NEB) twice and the column was washed twice with MTPBS buffer (150 mM NaCl, 16 mM Na$_2$HPO$_4$, 4 mM NaH$_2$PO$_4$, pH 7.3) before the bound protein was eluted with MTPBS plus 10 mM maltose. Five 1 mL aliquots were collected and the protein was quantitated by using the Bradford assay (BioRad). The first four aliquots were pooled and the final concentration of protein was 1 mg/mL. A 10% SDS-polyacrylamide gel was run with the following samples: the uninduced cells, induced cells, crude extract and column-purified sample. The gel was then Coomassie-stained. The pooled eluate in MTPBS was dialyzed against buffer containing 25 mM HEPES, 20%
glycerol, 100 mM NaCl, 10 mM β-mercaptoethanol, 10 mM maltose and the protein quantitated again by using the Bradford method, which indicated that virtually no protein was lost during the dialysis.

_Aqueous Coupling of Antigen to Affigel:_

The MBP-HER-1 sample was coupled to an Affi-Gel-15 (BioRad) matrix under aqueous conditions. Four mL of the MBP-HER-1-containing solution (1 mg/mL) was mixed with 5 mL of resin for two hours at room temperature. This was followed by another two hours of mixing at 4°C with 0.1 mL of 1 M ethanolamine pH 8. The slurry was then packed into a column and washed once with MTPBS. A Bradford assay was used to determine the amount of unbound protein in the wash and it was found that approximately 80 µg, or 2% of the input had not bound, indicating approximately 98% coupling efficiency.

_Purification of Antibodies:_

The column was pre-eluted with 10 mL of 100 mM glycine pH 2.5, 10 mL 100 mM glycine pH 11.5, and 10 mL MTPBS buffer. One mL of crude rat serum was diluted 1:10 in MTPBS and passed over the affinity column three times at 4°C. The column was washed with four bed volumes of 10 mM Tris-Cl pH 7.5 followed by four volumes 10 mM Tris-Cl, 500 mM NaCl, pH 7.5. The bound IgG was then eluted from the column with 1 mL of 100 mM glycine pH 2.5. The column was subsequently washed with aliquots of four bed volumes of 10 mM Tris-Cl pH 8.8 until the pass-through was pH 8.8 (five times) then eluted with 1 mL of 100 mM glycine pH 11.5. The column was finally washed with 10 mM Tris-Cl pH 7.5 until the pass-through was pH 7.5. The two eluates were
neutralized by addition of 1 M Tris-Cl pH 8 then pooled and concentrated by treatment in
Centricon-10 units.

**Testing Antibodies for Ability to Detect Secreted HER-1:**

To test the ability of the purified antibodies to detect secreted HER-1, I used cells that had
been transformed with a construct, pMG-1, encoding MBP-HER-1 with a secretion signal.
Expression of the fusion gene was induced and the cells were harvested and resuspended
in 100 mL of 30 mM Tris-Cl, 20% sucrose pH 8, 10 mM EDTA pH 8. The resuspended
cells were then shaken for 10 min. at room temperature. The cells were subsequently
centrifuged and resuspended in 100 mL of ice-cold 5 mM MgSO₄ before shaking for 10
min. at 4° C. Following centrifugation, the supernatant was collected and mixed with 2 mL
of 1M Tris-Cl pH 7.4. A Bradford assay revealed that the protein concentration of the
supernatant was ~200 µg/mL.

The MBP-HER-1 in 10 mL of the osmotic shock extract was purified by affinity
chromatography on a 2 mL amylose column. The column was pre-eluted and subsequently
washed with five bed volumes of MTPBS prior to loading the extract. The bound protein
was eluted with ten 1 mL aliquots of 10 mM maltose in MTPBS. The protein
concentration in these fractions was determined by using the Bradford assay. A total of
~540 µg of protein had been recovered in 7 mL. Twenty µL aliquots of each of the eluates
were run on a 10% SDS-polyacrylamide gel. The protein was transferred from the gel to a
0.2 µm nitrocellulose paper by semi-dry transfer procedure following standard techniques
(Sambrook *et al.*, 1989) and the blot was blocked for one hour at room temperature in
TBST (0.1 M Tris-Cl pH 8, 150 mM NaCl, 0.2% Tween-20) + 5% powdered milk. The blocked blot was then rocked overnight at room temperature in a solution containing the α-HER-1 antibodies diluted 1:3000 in TBST + 5% powdered milk in a seal-a-meal bag. This was followed by washing 3 x 10 min. at room temperature in TBST + 5% powdered milk and treatment with a solution containing a 1:20,000 dilution of peroxidase-conjugated α-rat antibodies in TBST + 5% powdered milk for two hours at room temperature. After washing again as described above, the ECL detection system (Amersham) was used and film (Kodak) was exposed to the blots for 20 min.

2.2 Expression of HER-1

Transfections:

Sf9 insect tissue culture cells were transfected with a baculoviral construct, pNR31, encoding full-length HER-1 with a 6 x histidine tag at its C-terminus, by using the Bac-N-Blue transfection kit (Invitrogen). By harvesting the supernatant and using it in repeated re-infections, the viral titre was increased to approximately 10^8 pfu/mL. The titre was measured by the end-point dilution assay wherein aliquots of 10 μL serial dilutions of the supernatant (10^-3, 10^-6, 10^-7 and 10^-8) were used to infect a dozen sets of 10^4 cells plated in separate wells of a 96-well plate and the number of wells exhibiting signs of infection were compared against the number which did not. Percentages of infected wells were calculated for each dilution.
Calculation:

Based on the Reed-Muench formula (Lennette and Schmidt, 1979), the proportionate distance (PD) of a 50% infection response from the response above 50% is \( PD = \frac{(A-50)}{(A-B)} \) where A is the % response above 50% and B is the % response below 50%. At \( 10^{-6} \) the % of infected wells was \(-70\% \) whereas at \( 10^{-7} \) it was \(-5\% \) so the PD = 0.3.

The dose that would have given a 50% response, the TCID\(_{50}\), is calculated as

\[
\text{Log TCID}_{50} = \log \left( \text{dilution giving response greater than } 50\% \right) - \text{PD of that response}
\]

The titre of the virus is the reciprocal of the TCID\(_{50} \) / 10 \( \mu \)L and can be converted to pfu/mL using the relationship pfu = TCID\(_{50} \times 0.69\). The TCID\(_{50} \) was found to be \( 10^{-6} \) and the titre was therefore calculated as \( 10^8 \) pfu/mL.

Testing for Expression:

To ensure that the infected Sf9 cells were secreting HER-1 into their growth medium, aliquots of the high-titre supernatant were electrophoresed on a 15% SDS-polyacrylamide gel which was subsequently Western blotted and probed with a solution containing a 1:3000 dilution of \( \alpha \)-HER-1 antibodies followed by a 1:20,000 dilution of peroxidase-conjugated \( \alpha \)-rat antibodies. Binding of the secondary antibody was visualized by use of an ECL system (Amersham).

The supernatant was then used to infect a 1 L culture of Sf9 cells which were growing in suspension and which had been adapted to growth in serum-free media, at an MOI of 10. Aliquots were taken from these cultures three, four and five days post-infection. Cells and
supernatant were separated by centrifugation and aliquots of each were then run on a 15% SDS-polyacrylamide gel. A Western blot of the gel was probed with a 1:3000 dilution of α-HER-1 antibodies and incubated with a 1:20,000 dilution of peroxidase-conjugated α-rat antibodies. To determine the concentration of HER-1 in the conditioned media, a known quantity of MBP-HER-1 was run alongside the sample for visual comparison.

**Dialysis:**

100 mL of the conditioned media was dialyzed against 4 L of a solution containing 10 mM sodium phosphate buffer pH 7, 40 mM NaCl, 0.02% sodium azide. The dialysis buffer was replaced eight times over a period of four days. 10 mL aliquots of the dialysate were separately adjusted to a final concentration of 300 mM NaCl, 50 mM sodium phosphate buffer pH 6, 6.6, 7, 7.6 or 8. Each was passed over a nickel affinity column (BioRad) in order to purify the His-HER-1 fusion protein. The columns were washed with 300 mM NaCl, 50 mM NaP, at the appropriate pH and the bound protein was eluted using the wash buffers containing 250 mM imidazole.

**Testing for Histidine Tag:**

Samples of the conditioned, serum-free medium and a 14.5 kDa histidine-tagged positive control (A. Rath, personal communication) were electrophoresed on a 15% SDS-polyacrylamide gel. Protein was transferred from the gel to a membrane and the membrane was treated with a 1:3000 dilution of rat antibodies directed against the polyhistidine epitope tag followed by incubation with a 1:20,000 dilution of peroxidase-conjugated α-rat antibodies. ECL detection was used.
2.3 Expression of TRA-2

Mammalian COS-7 cells were transfected with a construct called pMPH2-40, which is a GFP expression vector carrying the first 3799 base pairs of the tra-2 cDNA, under control of the strong CMV promoter (Figure 6). The GFP tag was utilized since antibodies against TRA-2 were not available. Transfections were performed using the CellPhect Kit (Pharmacia) Ca(PO₄)₂ method. Ten μg and 3 μg of maxiprepped (Qiagen) plasmid were used to transfect each 100 mm and 60 mm tissue culture plate of 70-80% confluent COS cells, respectively. Cells were processed for immunoblotting or examined under fluorescent microscopy 1-2 days post-transfection. As a control to test for generalized expression of GFP under control of the CMV promoter, cells were also transfected in parallel with the mammalian pEGFP expression vector (Clontech) into which I had cloned the strong CMV promoter. This was the parent plasmid to the pMPH2-40 construct (M. Perry, personal communication).

2.4 Testing for Binding of HER-1 to COS Cells Expressing TRA-2

Two different methods were used to test for binding of HER-1 to COS cells expressing TRA-2 on their surface. Following incubation with HER-1 conditioned media, these cells were either used in immunoblotting assays to test for the presence of HER-1 or were treated in immunoprecipitation assays to determine whether or not HER-1 was being co-immunoprecipitated.
Immunoblotting:

COS cells transfected with pMPH2-40 were incubated for 3-12 hours at 37°C with the HER-1 conditioned medium. Ten mL of conditioned medium containing 25 µg of HER-1 per mL was added to each 100 mm plate of transfected cells. As a control, non-transfected COS cells were treated in the same manner. Following the incubation period, the cells were gently scraped from the plates and centrifuged. They were then washed four times with 5 mL PBS and then resuspended in 50 µL of PBS containing 0.1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin and 1 µg/mL pepstatin.

25 µL of 50 mM dithiothreitol in Sample Buffer (NEB) was added to each sample and these were boiled for ten minutes before loading onto either a 6% or 15% SDS-polyacrylamide gel to test for expression of TRA-2::GFP or presence of HER-1, respectively.

Gels were Western blotted and blocked for 1 hour at room temperature, in TBST + 5% powdered milk. The blots were then incubated with either a 1:10,000 dilution of chicken α-GFP antibodies (Chemicon) to detect TRA-2::GFP or a 1:3000 dilution of α-HER-1 antibodies followed by a 1:10,000 dilution of peroxidase-conjugated α-chicken antibodies or 1:20,000 peroxidase-conjugated α-rat antibodies, respectively. Protein bands which interacted with the primary antibody were visualized using ECL detection.
Immunoprecipitation:

Untransfected and pMPH2-40-transfected COS cells were incubated for 3-12 hours at 37°C with HER-1 conditioned medium and then scraped off the plates and washed three times with 5 mL PBS, as described in section 2.4. They were resuspended in 1 mL of PLC lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM PMSF), vortexed vigorously and incubated for 1 hour on ice. The cellular debris was separated by centrifugation at 12,000 rpm for 20 min. and the supernatant was incubated on a rocking platform overnight at 4°C with 2 μg α-GFP antibodies and 10 μL protein A sepharose. The beads were then centrifuged out by spinning at 12,000 rpm for 1 min. and washed three times with 1 mL aliquots of PLC lysis buffer before being resuspended in 25 μL of 50 mM dithiothreitol in Sample Buffer (NEB), boiled and loaded onto either a 6% or 15% SDS-polyacrylamide gel. After Western blotting, the 6% gel was probed with a 1:10,000 dilution of α-GFP antibodies while the 15% gel was probed with a 1:3000 dilution of α-HER-1 antibodies. α-chicken and α-rat peroxidase enzymes were directed against the α-GFP and α-HER-1 primaries, respectively, and ECL detection was used.

2.5 Immunofluorescence

COS cells plated on acid-treated coverslips in 60 mm plates and transfected with 3 μg pMPH2-40 were incubated with 5 mL HER-1 conditioned media for 3-12 hours at 37°C. As controls, wildtype COS cells were treated in the same manner, and pMPH2-40
transfected COS cells were incubated with non-conditioned Grace's insect tissue culture media.

The media was aspirated and the cells washed 2 x 5 min. with 5 mL PBS then incubated for 1 hour at room temperature with 2 mL fixing solution (2% paraformaldehyde in 0.1M phosphate buffer pH 7.4). They were then washed 3 x 5 min. in 5 mL PBS and blocked for 1 hour at room temperature with 5 mL of 30% donkey serum (Sigma) in PBS. The cells were subsequently washed 2 x 5 min. in 5 mL PBS with 1% BSA (Sigma). 50 μL of 1:10 α-HER-1 antibodies in PBS, 1% BSA was aliquoted onto each coverslip of cells and the coverslips were covered with parafilm before incubating overnight at room temperature.

After incubation, the coverslips were washed 3 x 15 minutes in PBS containing 1% BSA and incubated for two hours at room temperature with 50 μL of a 1:50 dilution of rhodamine-conjugated α-rat secondary antibodies in PBS containing 1% BSA. They were then washed 3 x 15 minutes with PBS containing 1% BSA. The second wash solution also included 1 μg/mL DAPI, since the DNA-fluorochrome facilitates identification of the cells when viewed under a microscope using epifluorescent illumination. The coverslips were subsequently dotted with 10 μL aliquots of n-propylgallate (200 μg/μL) and mounted onto slides for viewing under the fluorescent microscope.
3. Results

The experiments outlined here were carried out to test for a direct interaction between the *C. elegans* sex determination gene products HER-1 and TRA-2. The initial goal was to obtain the reagents with which to test the proposed interaction. For this purpose, I purified antibodies directed against HER-1 for use in detecting the protein in subsequent immunoblotting, immunofluorescence and immunoprecipitation experiments. I obtained HER-1 protein by transfecting Sf9 insect tissue culture cells with a baculoviral construct encoding histidine-tagged full-length HER-1. The secreted protein, which was in the tissue culture medium, was used in binding assays with mammalian COS-7 cells which were transfected with a construct encoding the N-terminal extracellular domain and transmembrane portions of the TRA-2 protein plus a GFP tag.

3.1 HER-1 Expression from Sf9 Insect Tissue Culture Cells

I verified that the rat α-HER-1 antibodies could detect HER-1 in a Western blot immunodetection system (see Materials and Methods). MBP-HER-1 produced in bacteria was run on an SDS-polyacrylamide gel and the protein was transferred to a membrane (see Materials and Methods). The membrane was treated with affinity-purified rat α-HER-1 antibodies and bound antibody was detected by use of a peroxidase-conjugated α-rat secondary antibody and an ECL detection system. The purified α-HER-1 antibodies were able to detect secreted 60 kDa MBP-HER-1 fusion protein when diluted 1:3000 and probed with peroxidase-tagged α-rat antibodies diluted 1:20,000 (Figure 7). Lower and higher dilutions of the primary antibody were tested but 1:3000 proved optimal for
detection with minimal background. This purified antibody was used in subsequent immunoblotting and immunofluorescence experiments.

I subsequently used the affinity-purified antibodies to test whether Sf9 cells transfected with the her-1 baculoviral construct were secreting HER-1 protein into their growth medium. A Western blot of the cellular supernatant was probed with the α-HER-1 antibodies, which detected the presence of an approximately 20 kDa protein (Figure 8). This measurement corresponds to the expected size of the HER-1 protein, and thus suggests that the infected cells were expressing HER-1 and secreting it into their growth medium. As a positive control, 2 and 5 μg aliquots of the purified and quantitated 60 kDa MBP-HER-1 fusion protein were run alongside the two independent supernatant samples for comparison. In each case the HER-1 was detected as a doublet and this is characteristic of the protein (M. Perry, personal communication).

An estimate of the HER-1 concentration in the growth medium was made based on comparison with the aliquots of MBP-HER-1. The signal from the 20 μL supernatant samples appeared to be approximately four-fold lower than that produced by the 2 μg aliquot of MBP-HER-1. Based on that assumption, I calculated that the concentration of HER-1 in the growth medium was approximately 25 μg/mL.

For large-scale production of HER-1, the supernatant from these transfection experiments was used to infect a suspension culture of Sf9 cells growing in serum-free medium (in order to eliminate contamination by the bovine serum albumin in fetal bovine serum during
subsequent purification steps). Aliquots of the cells and supernatant were taken and separated on days three, four and five post-infection. Probing the blot of these samples with α-HER-1 antibodies indicated that the 20 kDa protein was being expressed in the cells by day three and being secreted into the supernatant by day four (Figure 9). The increased amount of HER-1 in the cells by days three and four caused an artifact in which the antibody appears to be excluded from the HER-1 bound by the blot. This accounts for the clear signal at ~20 kDa in these lanes. Another signal of approximately 65 kDa appeared to correspond with the presence of HER-1 in both the cells and supernatant in each case. This signal correlated with infection of the cells as it did not appear in the non-infected control. I intended to ascertain whether this unknown cross-reacting product could be eliminated by subsequent purification of the histidine-tagged HER-1 over a nickel affinity column, as would likely be the case if it were a viral or cellular product resulting from the transfection. However, as described below, this was not possible.

I next endeavored to utilize the histidine tag that I expected to be present on the HER-1 protein, to purify it on a nickel column. To this end, I obtained more HER-1 secreted from infected Sf9 cells and dialyzed the protein into a phosphate buffer to facilitate adhesion to the nickel resin. The dialyzed protein was then diluted in a set of buffers ranging in pH from 6.0 to 8.0, given that the predicted pI of HER-1 is ~8.8 (ExPASy Compute pI/MW Tool). Protein preparations at each of these pHs were passed over the nickel column, and the columns were then washed and eluted under the appropriate conditions. In each case I found that the protein was present only in the flow-through and none of it appeared to
bind to the matrix, as assessed by electrophoresis of the flow-through, washes and eluates on an SDS-polyacrylamide gel and Coomassie staining of the gel.

To determine the source of this problem, a Western blot of the conditioned media which had not been dialyzed was probed with a-histidine rat antibodies and peroxidase-tagged a-rat antibodies. As a positive control, an equivalent amount (~0.5 μg) of a 14.5 kDa histidine-tagged protein preparation was run as well and it was found that while the antibodies detected this control protein, no signal was obtained in the conditioned medium sample (Figure 10). This indicated that the histidine tag was therefore absent from the HER-1 in this supernatant. The baculoviral construct used to transfect the Sf9 cells was sequenced by N. Rajwans (personal communication) and it was found that the sequence encoding the histidine tag had indeed been cloned in-frame. This suggested that the tag was being cleaved off at some time during the processing of the protein in this cell system. Since the lack of a histidine tag prevented purification of HER-1 on the nickel column, all subsequent experiments utilized the original conditioned media.

3.2 TRA-2 Expression in Mammalian COS Cells

My initial attempt to test for a TRA-2-HER-1 interaction in tissue culture cells was done by expressing TRA-2 in Sf9 cells. I used a baculoviral expression vector, pBlueBac III (Invitrogen), carrying the full-length trα-2 cDNA with a sequence encoding an HA epitope tag cloned into the 3’ end of the cDNA (N. Rajwans, personal communication). This plasmid, NR28, was transfected into Sf9 insect cells and its titre increased to ~10^7 pfu/mL. The transfected cells were used in a binding assay with the HER-1 conditioned medium.
However, in attempting to assess localization of TRA-2::HA using mouse antibodies directed against the HA tag (M. Tyers, personal communication) and FITC-labeled α-mouse secondary antibodies, I found that the nuclei of these cells tended to swell upon infection with the baculovirus. This made it difficult to determine exactly which portion of the cell was fluorescing. I therefore abandoned the use of Sf9 cells.

Subsequent experiments attempted to make use of mammalian COS cells as an alternative to Sf9 cells. The construct used in these experiments consisted of the pcDNA-3 mammalian expression vector (Invitrogen) with the full-length tra-2 cDNA and a sequence encoding an HA epitope tag at the 3' end of the cDNA (N. Rajwans, personal communication). This plasmid, NR35, was transfected into COS cells which were then examined for expression of the fusion protein by both immunoblotting and immunofluorescence. In each case, mouse α-HA antibodies were directed against the epitope tag. A peroxidase-tagged α-mouse antibody (Boehringer Mannheim) was used on the Western blots while FITC-labeled α-mouse antibodies were used in the immunofluorescence assay. However, full-length TRA-2::HA could not be detected from these cells in the immunoblotting experiments. Modifications to the protocol were attempted. For example, transfection with increased concentrations of NR35 was tested and cells were lysed with 2% Triton-X 100 and treated at 60° C (rather than boiling), but none of these proved fruitful in allowing detection of the ~170 kDa fusion.

The initial immunofluorescence assay indicated bright green color in the interior of some of the cells. This expression did not appear localized to either the membrane or nucleus.
but rather seemed spread out through the cell cytoplasm. These results, however, were not reproducible. At this point I decided to use a GFP-tagged version of TRA-2 which would circumvent the need for using antibodies to detect TRA-2 in the immunofluorescence assay. A plasmid carrying a \textit{tra-2} minigene followed by an in-frame GFP expression cassette was subsequently constructed (M. Perry, personal communication). This construct contained the portion of the \textit{tra-2} cDNA which encodes the transmembrane domains of TRA-2 thought to be responsible for anchoring the protein to the membrane and the N-terminal, extracellular portion which is the proposed site of interaction with HER-1.

All subsequent experiments were therefore performed using the TRA-2::GFP construct. A Western blot of COS cells transfected with the construct encoding TRA-2::GFP and non-transfected cells was probed with $\alpha$-GFP antibodies. Subsequent immunodetection by use of an ECL system indicated that an approximately 142 kDa protein was present in the transfected cells, but not in the non-transfected COS cells (Figure 11). Calculations of the predicted molecular weight of the protein encoded by the portion of the \textit{tra-2} sequence inserted into the TRA-2::GFP construct plus the $\sim$27 kDa GFP tag (Clontech) corresponded with this size (ExPASy Compute pI/MW Tool).

Fixed COS cells transfected with this TRA-2::GFP construct and untransfected controls were viewed by fluorescence microscopy using the FITC channel. As shown in Figure 12a, the wildtype cells appeared to be pale green under these conditions. On the other hand, the transfected cells, shown in Figure 12b, showed a ring of brighter yellow-green around the
perimeter of the cells and as well near the centre of the cells. Calculations of the total number of cells on a single slide expressing the brighter yellow-green fluorescence compared to those which did not indicated a transfection efficiency of approximately 52% (Table 1).

3.3 HER-1 and TRA-2 Interactions

Two complementary assays were used to ask if the transiently transfected COS cells expressing TRA-2::GFP on their surface were competent to bind to recombinant HER-1 protein: immunoblotting and immunofluorescence assays. A third approach, immunoprecipitation, proved unsuccessful in addressing this question.

*Immunoblotting:*

When probed with α-HER-1 antibodies, a Western blot of COS cells transfected with the TRA-2::GFP construct and incubated with HER-1 conditioned medium indicated that HER-1 protein was present in the lane containing the transfected cells but not the non-transfected COS cells treated in the same fashion (Figure 13, Lanes 1 and 2). This result, however, should be interpreted cautiously because the HER-1 detected in the experimental lane (Lane 1) gives a minimal signal. This makes it possible that a signal in the negative control (Lane 2) may have escaped detection. Thus, one cannot exclude the possibility that the signal in Lane 1 may reflect HER-1 non-specifically trapped in the cell pellet.
As a positive control, 20 µL of conditioned media was loaded as well. To estimate the amount of HER-1 bound to the transfected cells, visual comparison to the positive control can be used in calculations as follows.

**Calculations:**

\[
(20 \, \mu\text{L}) \times (25 \, \mu\text{g/mL} \, \text{HER-1 in conditioned media}) / 1000 \, \mu\text{L/mL} = 0.5 \, \mu\text{g} \, \text{HER-1 in positive control lane}
\]

Approximately ten times more signal from positive control compared to sample thus:

\[
\sim 0.05 \, \mu\text{g} \, \text{HER-1 bound}
\]

Given that the molecular weight of HER-1 is 20 kDa, which is 20,000 g/mol:

\[
\# \, \text{of moles of HER-1} = 0.05 \, \mu\text{g} / (10^6 \, \mu\text{g/g})(20,000 \, \text{g/mol})
\]

\[
= 2.5 \times 10^{12} \, \text{moles of HER-1 bound}
\]

Therefore the number of molecules of HER-1 bound is:

\[
(2.5 \times 10^{12} \, \text{moles}) \times (6.022 \times 10^{23} \, \text{molecules/mole})
\]

\[
= 1.5 \times 10^{12} \, \text{molecules of HER-1 bound per plate}
\]

Given that it was estimated approximately 52% of cells were transfected and that there were approximately 10^7 cells plated on the dish in which the experiment was performed, this indicates that the \( \sim 5.2 \times 10^6 \) cells expressing TRA-2::GFP were able to bind approximately \( 1.5 \times 10^{12} \) molecules of HER-1. Thus it can be deduced that \( \sim 2.9 \times 10^5 \) molecules of HER-1 bound per cell expressing the TRA-2::GFP fusion. This number correlates well with what would be expected based on the assumption of a one to one ratio of ligand bound to receptor (Huang *et al.*, 1990).
Immunoprecipitation:

To provide further evidence supporting the hypothesis that the TRA-2-dependent adhesion of HER-1 from conditioned medium seen in the immunoblotting and immunofluorescence assays was indeed evidence of a direct interaction between the two proteins, I tested whether HER-1 would co-immunoprecipitate with membrane-bound TRA-2::GFP using chicken α-GFP antibodies.

Membranes of COS cells transfected with pMPH2-40 were solubilized with or without pretreatment with HER-1 conditioned media. The solubilized membranes were then treated with chicken α-GFP antibodies. Non-transfected COS cells were used as controls in each case. I was, however, unable to detect the TRA-2::GFP on a Western blot of the sepharose beads used in the immunoprecipitation assay (Figure 13, Lanes 3 and 4). At this time, however, I learnt that protein A has very low affinity for chicken antibodies. A future approach would be to use an intermediary antibody with affinity for the chicken antibodies as well as protein A interaction of the α-GFP antibodies with the protein A sepharose beads.

Immunofluorescence:

The second assay that I used took advantage of immunofluorescence techniques and involved the COS cells which had been transfected with the TRA-2::GFP construct and incubated with conditioned medium containing HER-1. A rhodamine-conjugated secondary antibody was used to detect any HER-1, which may adhere to the COS cells.
expressing TRA-2. If indeed TRA-2 were a membrane-localized receptor for HER-1, I would expect that fluorescence corresponding to the rhodamine-labeled secondary antibody, directed against the α-HER-1 antibodies, would co-localize with fluorescence corresponding to the expression of TRA-2::GFP.

When viewed using epifluorescence and $\lambda = 535$ nm light, transfected cells that had been incubated with HER-1 followed by rat α-HER-1 antibodies and rhodamine-labeled α-rat secondary antibodies, appeared to show bright red fluorescence under the rhodamine channel around the periphery and centre of the cells expressing bright green GFP fluorescence in these same regions of the cell (Figure 14a, b). It was found that the bright red co-localized with the bright green pattern in ~95% of the cells (Table 2). A possible explanation for the failure to detect co-localization of red fluorescence on ~5% of the cells may be that fewer TRA-2 receptors were expressed on these particular cells, resulting in a lower concentration of HER-1 and therefore both α-HER-1 and rhodamine-conjugated α-rat antibodies. The amount of fluorochrome interacting with such cells may not have been sufficient to produce adequate signal for visual detection using epifluorescent illumination.

To determine whether the rhodamine-labeled secondary antibody recognized any non-specific epitopes on the surface of the transfected COS cells (demonstrating bright green fluorescence corresponding to TRA-2::GFP expression), fixed cells which had not been treated with the HER-1 conditioned medium were incubated with the rhodamine-conjugated secondary antibody, washed and viewed using epifluorescence and $\lambda = 450 - 490$ nm light. In parallel, fixed COS cells which had not been treated with the secondary
antibody were also mounted for microscopy. No red signal could be detected on either control sample. It could therefore be concluded that the commercially-prepared rhodamine-labeled α-rat secondary antibodies do not adhere to COS cells under these conditions. As a further control, it may prove useful to repeat this experiment in the absence of the primary antibodies as well.
4. Discussion

The primary goal of this project was to test the hypothesis that a direct physical interaction takes place between HER-1 and TRA-2. To address this issue, the reagents required to test the proposed interaction needed to be obtained. To that end, antibodies directed against HER-1, a source of HER-1 protein and cells expressing TRA-2 on their surface were prepared. Immunoblotting, immunofluorescence and immunoprecipitation experiments were subsequently performed using these reagents, to determine whether HER-1 could adhere to the surface of COS cells expressing a TRA-2 fusion protein, and whether this interaction could survive the solubilization of the TRA-2 fusion from the membranes of these cells.

The preparation of the reagents initially involved addressing whether or not HER-1 could be expressed in insect cell culture, secreted from these cells into their media, and whether or not this could be achieved on a large scale. Secondly, the issue of TRA-2 expression needed to be taken into consideration. Could TRA-2 be expressed in tissue culture? And if so, how would it be detected, given the lack of antibodies specific to the protein? The results presented in this thesis demonstrate that HER-1 can be expressed and secreted from Sf9 insect tissue culture cells in large scale (1 L) cultures and that this secreted protein can be detected using the purified α-HER-1 antibodies. Further, I have shown that a TRA-2::GFP fusion protein can be expressed in mammalian COS-7 cells, permitting ready detection using either α-GFP antibodies on Western blots or using epifluorescent illumination of live and fixed cells with λ = 450 - 495 nm light.
Given the reagents at hand, it was then possible to address the question of the proposed HER-1/TRA-2 interaction. There were a number of possible approaches to this issue, among which were:

i) cross-linking and immunoprecipitation of radiolabeled HER-1 to COS cells expressing TRA-2::GFP

ii) immunofluorescence assays using fluorescent antibodies to detect any HER-1 which may co-localize with GFP expression in COS cells expressing TRA-2::GFP

iii) immunoblotting assays to detect HER-1 on COS cells expressing TRA-2::GFP

iv) affinity chromatography to test for binding of HER-1 to immobilized TRA-2::GFP

v) scintillation proximity assays wherein purified TRA-2::GFP can be linked to scintillation proximity assay beads and binding of radiolabeled HER-1 can be measured without separation of bound from free ligand

Based on the reagents available and limitations of the system, I initially selected immunofluorescence, immunoblotting and immunoprecipitation assays (without radiolabeled HER-1) to investigate the proposed interaction.

4.1 TRA-2::GFP Expression Pattern

The fluorescence detected predominantly around the periphery of the COS cells transfected with the TRA-2::GFP construct corresponds with the predicted expression pattern of TRA-2, based on its alternating hydrophobic and hydrophilic domains which suggest that it is a transmembrane protein. The bright green fluorescence at the interior of
the cells could result from processing of the fusion protein through the interior components of the cell such as the endoplasmic reticulum, endosomes or the golgi apparatus.

4.2 HER-1 and TRA-2 Interactions:
The experiments presented here demonstrate the feasibility of expressing the *C. elegans* protein TRA-2 in vertebrate cell culture and that the *C. elegans* HER-1 protein can be successfully expressed and secreted from insect tissue culture cells.

Co-localization of HER-1 and TRA-2::GFP on COS cells is consistent with a direct interaction between the two proteins and implies that the existence of a *C. elegans* adapter protein mediating the interaction between HER-1 and TRA-2 is unlikely. The results do not, however, rule out the possibility that there may be a factor in the tissue culture media or on the cells which may have facilitated the interaction. The co-immunoprecipitation experiment designed to test for direct interactions, unfortunately, failed for technical reasons.

As an extension of the cell binding and co-immunoprecipitation experiments performed to test the proposed interaction, it would be interesting to test the ability of HER-1 to interact with a TRA-2 protein carrying the enhanced gain-of-function mutation. As discussed earlier, the R177K point mutation known as the *tra-2(eg)* allele is insensitive to negative regulation by *her-1(gf)* alleles on a genetic level (Hodgkin and Albertson, 1995; Kuwabara, 1996). If the hypothesized interaction between the two proteins is indeed
direct, then one would expect that this version of TRA-2 would fail to sequester HER-1 in binding assays and in immunoprecipitation experiments.

To that end, I have cloned a 2.2 kb portion of the tra-2 cDNA which spans the eg site, into the pBluescript II SK(-) vector (Stratagene) in order to carry out site-directed mutagenesis. This mutated 2.2 kb portion of the cDNA can be cloned into the TRA-2::GFP construct to replace the wildtype eg site.

Another control experiment to consider is the overexpression of a non-relevant membrane protein as a non-specific competitor to TRA-2. To examine whether or not the suggested interaction is specific or if HER-1 is simply attaching randomly to surface proteins, COS cells could be transfected with a construct encoding such a protein and incubated with HER-1. This can be tested in both immunoblotting and immunofluorescence binding assays as well as in the proposed immunoprecipitation experiments.

With regard to the HER-1 protein's ability to stick to cells transfected with the TRA-2::GFP construct, serial dilutions of the conditioned media need to be tested to assess the minimal amount required for the interaction to be detected in both immunoblotting and immunofluorescence assays.

4.3 Further Experiments

To quantitate the strength of the proposed interaction, the dissociation constant ($K_D$) needs to be measured. It is thus necessary to determine the ratio of unbound and bound
HER-1 at varying concentrations of ligand while the amount of TRA-2 remains constant. To do this, the HER-1 protein can be radiolabeled then incubated with COS cells expressing TRA-2::GFP and any bound radioactivity can be counted. To ascertain whether the hypothesized interaction is specific, unlabeled HER-1 can be used as a competitor. α-HER-1 antibodies can also be used to diminish binding and therefore demonstrate the specificity of the interaction.

Another possibility would be to use affinity chromatography, wherein TRA-2 can be purified from the membrane fraction of transfected COS cells and coupled to a resin over which HER-1 can be passed and tested for binding.

In order to map the regions of HER-1 that are necessary for the proposed interaction to take place, truncated versions of the protein may be used in the aforementioned assays as unlabeled competitors. To map the portions of TRA-2 which are required for the suggested interaction, N-terminal-, internally- and C-terminal-deleted versions of the protein can be examined for their ability to bind HER-1 using the various assays discussed above.

On a related note, it may prove valuable to examine functional conservation on an evolutionary level. HER-1 homologues from *C. elegans* and the related nematode species *C. briggsae* and *B. malayi* (Streit et al., 1999) could be tested for physical interaction with the various TRA-2 homologues which have been cloned from *C. elegans*, *C. briggsae* and *C. remanei* (Haag and Kimble, 2000; Kuwabara and Shah, 1994). Binding of the foreign
proteins to a particular site could uncover conserved residues which may be important in facilitating the proposed interaction between the two proteins.

The experimental results presented here provide evidence in favor of a physical interaction between HER-1 and TRA-2, a hypothesis which is based on the genetic data presented to date. The purported ligand-receptor interaction can be incorporated into a signal transduction pathway, as outlined by the proposed molecular interactions shown in Figure 4. The binding of HER-1 to TRA-2 in the XO cell may be the primary signal initiating the male developmental pathway within that particular cell. This signal could subsequently be transmitted to the transcriptional machinery within the nucleus via the FEM proteins and the terminal regulator, TRA-1, which is prevented from activating transcription of hermaphrodite-specific genes. In the absence of TRA-1 activity, the cell is permitted to adopt the male fate.

Furthermore, this interaction ties in with the observed cellular non-autonomy of HER-1 (Hunter and Wood, 1992). The fact that expression of her-1 from one set of cells can recruit neighboring her-1(-) cells to the male fate suggests that HER-1 protein secreted from the her-1(-) cells is capable of somehow inducing the transcriptional apparatus of her-1 mutant cells to express male-specific genes. Under the proposed model, it would follow that the HER-1 secreted from her-1(-) cells interacts physically with TRA-2 on the surface of her-1(-) cells, thereby initiating the signal transduction pathway which culminates in repression of transcription of hermaphrodite-specific genes. Thus, HER-1
could be viewed as a molecule which mediates cell-to-cell communication in *C. elegans* sex determination, promoting the adoption of the male fate.

Overall, the evidence presented here supports a direct physical interaction between HER-1 and TRA-2, providing insight into the molecular mechanism by which sex determination takes place within an individual cell during *C. elegans* development. This in turn may afford one potential response to the question posed in developmental biology regarding the manner in which cells establish themselves as one of two dimorphic sexes in eukaryotic organisms. The series of protein-protein interactions outlined by the proposed molecular model present a route permitting the transmission of a signal, originating from the expression and/or repression of a particular gene or genes, resulting in the specification of a cell’s sexual identity.

While further experiments are needed to confirm the hypothesis, the molecular data presented in this thesis are encouraging and provide a basis for further exploring the suggested interaction between HER-1 and TRA-2.
Transformation Efficiency of TRA-2::GFP Construct in COS Cells

<table>
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Table 1
**Correlation of GFP and Rhodamine Expression in Immunofluorescence Assay**

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Table 2
Figure 1: Sexual Dimorphism in *C. elegans*

Wildtype XX *C. elegans* animals develop as hermaphrodites (top) whereas wildtype XO animals develop as males (bottom). Major anatomical features of each sex are indicated (Sulston and Horvitz, 1997).
Figure 2: Regulation of Somatic Sex Determination and Dosage Compensation

Pathway outlining epistatic interactions between characterized genes involved in *C. elegans* dosage compensation and sex determination. Barred lines indicate negative interactions while arrows demote positive interactions. The primary X:A ratio controls dosage compensation and sex determination. These two branches of the pathway diverge after the *sdc* genes. (Doniach and Hodgkin, 1984; Hodgkin, 1980; Hodgkin, 1986; Miller *et al.*, 1988; Villeneuve and Meyer, 1987).
Dosage compensation

*dpy-26, dpy-27, dpy-28*

Sex determination

*Figure 2*
Figure 3: Physical Map of the her-1 Locus

The her-1 gene is depicted as a horizontal line. Two male-specific promoters, P1 and P2, are indicated. Filled boxes indicate exons while open boxes represent the 5’ and 3’ untranslated regions of the terminal exons. The sequence of the locus is indicated in the lower portion of this figure, with the loss of function and gain of function sites outlined. Figure adapted from Perry et al. (1993).
**Figure 4: Molecular Model for Somatic Sex Determination**

Diagram prepared by Dr. Jeb Gaudet

The proposed protein-protein interactions involved in the specification of *C. elegans* somatic sex for both the XX cell (top) and the XO cell (bottom). Direct physical interactions represented here have been demonstrated *in vitro*. As the role of TRA-3 is not clear it is not included in this diagram.

In the XX cell, TRA-2 is able to bind FEM-3 thereby inhibiting it from interacting with the other FEM proteins and permitting TRA-1 to activate transcription of hermaphrodite-specific genes.

In the XO cell, interaction with HER-1 is proposed to induce a conformation of TRA-2 which prevents it from sequestering FEM-3 and thus allowing the FEM proteins to cooperate in down-regulating TRA-1 activity.
The **XX Cell**

Extracellular Space

Cytoplasm

Nucleus

---

The **XO Cell**

Extracellular Space

Cytoplasm

Nucleus

---

*Figure 4*
Figure 5: Germline Sex Determination

The epistatic interactions between *C. elegans* sex determination genes is somewhat different in the germline than in the soma. Barred lines indicate negative interactions while arrows indicate positive interactions. Figure adapted from Cline and Meyer (1996).
Figure 5
Figure 6: Plasmid Map of pMPH2-40

Construct encoding TRA-2::GFP fusion from the strong CMV promoter. Parent plasmid is pEGFP (Clontech) which directs enhanced GFP expression in mammalian cells.
Figure 6
Figure 7: Testing for Antibody Function

Western blot analysis of three fractions of MBP-HER-1 that had been purified from bacteria subject to SDS-PAGE. The blot was probed with rat anti-HER-1 antibodies followed by peroxidase-tagged anti-rat secondary antibodies. ECL detection was used. Fusion protein was detected as a doublet at approximately 60 kDa.
Figure 7
Figure 8: Recombinant HER-1 Expression in Tissue Culture Cells

Western blot of SDS-PAGE:

Lane 1 contains a 2 $\mu$g sample of purified MBP-HER-1 protein.

Lane 2 contains a 5 $\mu$g sample of purified MBP-HER-1 protein. MBP-HER-1 is detected as a doublet at approximately 60 kDa.

Lanes 3 and 4 contain two separate aliquots of growth medium taken from Sf9 insect cells infected with a baculoviral construct encoding the HER-1 protein with a secretion signal and 6 x histidine tag at its N-terminus. The HER-1 protein is detected at approximately 20 kDa. The blot was probed with rat anti-HER-1 antibodies and peroxidase-tagged anti-rat secondary antibodies. ECL detection was used.
Figure 8
Figure 9: Time Course of Recombinant HER-1 Expression from Sf9 Insect Cells

Western blot of SDS-PAGE:

Supernatant + positive control
Cell Pellet - negative control

Sf9 insect cells grown in serum-free medium and infected with a baculoviral construct encoding the HER-1 protein with a secretion signal and 6x histidine tag at its C-terminus. Cells and supernatant were run on the denaturing gel. The blot was probed with rat anti-HER-1 antibodies and peroxidase-tagged anti-rat secondary antibodies. ECL detection was used.

Lanes 1, 3 and 5 contain supernatant from days 5, 4 and 3 post-infection, respectively. Signal corresponding to ~20 kDa HER-1 is detected in day 4 and day 5 samples.

Lanes 2, 4 and 6 contain cells from days 5, 4 and 3 post-infection, respectively. Signal corresponding to ~20 kDa HER-1 is detected in all three samples.

Lane 7 contains the original aliquot of infective supernatant used to inoculate the above culture and signal corresponding to ~20 kDa HER-1 is detected.

Lane 8 contains MBP-HER-1 fusion protein as a positive control and signal corresponding to the ~60 kDa fusion is detected.

Lane 9 contains supernatant from day 5 of wildtype cells grown in serum-free media and no signal is detected as expected for this negative control.
Figure 9
Figure 10: Absence of Signal Corresponding to the Histidine Tag

Western blot of SDS-PAGE with positive control for histidine tag and HER-1 conditioned media. Blot was probed with rat anti-histidine antibodies and peroxidase-tagged anti-rat secondary antibodies. ECL detection was used.

Lane 1 contains a 14.5 kDa histidine-tagged protein while Lane 2 contains 20 μL of the HER-1 conditioned media. The positive control is detected by the antibodies directed against the histidine tag but the HER-1 from the conditioned media is not, indicating that the epitope tag is either absent or inaccessible.
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Figure 10
Figure 11: Expression of TRA-2::GFP

Western blot of SDS-PAGE with non-transfected and transfected COS cells probed with chicken anti-GFP antibodies and peroxidase-tagged anti-chicken peroxidase secondary antibodies. ECL detection was used.

Lane 1 contains COS cells transiently transfected with pMPH2-40 while Lane 2 contains wildtype COS cells. Signal corresponding to ~142 kDa was detected in the transfected cells but not in the non-transfected cells.
Figure 12: Localized Expression of TRA-2::GFP

Fluorescent images of COS cells which were fixed to coverslips and DAPI-stained.

(a) Wildtype COS cells viewed under the FITC channel of a fluorescent microscope at 200x magnification. Establishes the level of fluorescence which can be attributed to background alone.

(b) COS cells transiently transfected with the pMPH2-40 construct encoding TRA-2::GFP and viewed under the FITC channel of a fluorescent microscope at 200x magnification. Cells exhibit brighter color around the perimeter as well as at the centre.
Figure 13: TRA-2-Dependent Binding of HER-1 to COS Cells

Western blot of SDS-PAGE:

Wildtype COS cells (Lanes 2 and 4) and COS cells transiently-transfected with pMPH2-40 corresponding to TRA-2::GFP expression (Lanes 1 and 3) were incubated with serum-free HER-1 conditioned media. Cells were collected and processed as described in Materials and Methods.

Lane 5 contains 20 μL of HER-1 conditioned medium as a positive control and signal corresponding to the ~ 20 kDa HER-1 is detected.

Western blot was probed with rat anti-HER-1 antibodies and peroxidase-tagged anti-rat secondary antibodies. ECL detection was used.
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Figure 13
Figure 14: Co-Localization of HER-1 and TRA-2 Demonstrated by Immunofluorescence

Fluorescent images of COS cells which have been transiently transfected with the TRA-2::GFP construct and incubated with HER-1 conditioned media. The cells were fixed, blocked with donkey serum and treated with rat anti-HER-1 antibodies and rhodamine-conjugated anti-rat antibodies.

(a) Transfected COS cell viewed under the FITC channel of a fluorescent microscope at 200x magnification and expressing brighter color around the perimeter and at the centre.

(b) Same cell as shown in (a) viewed under the rhodamine channel and exhibiting bright red color in the same regions as bright green was seen under the FITC channel.
References


